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Potential roles for transforming growth factor-B during terminal differentiation of ocular lens cells /

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POTENTIAL ROLES FOR TRANSFORMING GROWTH FACTOR-β DURING TERMINAL DIFFERENTIATION OF OCULAR LENS CELLS

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Introduction

INTRODUCTION

The ocular lens continues to grow in size throughout the life of an organism. The increase in size is due to the continual addition of lens fiber cells to the periphery of the lens. The lens fibers originate as the progeny of epithelial cells located within the germinative zone, a ring of mitotic cells located on the anterior surface of the lens. The post-mitotic daughter cells begin terminally differentiating into lens fiber cells as they migrate along the lens capsule towards the lens equator. Once beyond the lens equator, the lens cells rotate their apical-basal axis 180° and elongate into mature fiber cells which are packed as concentric shells atop the existing lens fiber cells. Throughout terminal differentiation, the lens cells will remain withdrawn from the cell cycle; eliminate all intracellular organelles; synthesize increased amounts of lens-specific crystallins, cytoskeletal and membrane proteins; and become intimately associated with their neighboring lens fiber cells (Persons and Modak, 1970; Kuwabara, 1975).

Fiber cell terminal differentiation may be under the control of genetic mechanisms or morphogenic cues located within the microenvironments which encompass the lens cells. During the migration of the differentiating fiber cells, their basal surface remains in contact with the lens capsule. The lens capsule, the continuous basement membrane that surrounds the lens, differs in thickness, composition and architecture between the anterior and the posterior surface (Kuwabara, 1975; Johnson and Beebe, 1984; Mohan and Spiro, 1986; Webster Jr., 1987). The lens capsule is also bathed in different ocular solutions; the anterior surface contacts the aqueous humor while the posterior side is covered with vitreous humor. The aqueous and vitreous humors vary in their ability to influence lens cell behavior (Coulombre

and Coulombre, 1963; Lovicu, Chamberlain and McAvoy, 1995). Thus, the heterogeneity existing between the different regions of the lens capsule could be the result of soluble matrix-degrading enzymes preferentially found within the aqueous or vitreous humors. Alternatively, the differences found within the lens capsule could result from the preferential production/modification of the capsule as it is being deposited by the lens cells themselves (Young and Ocumpaugh, 1966; Rafferty and Goossen, 1978; Fitch, Mayne and Linsenmayer, 1983; Laurent, et al., 1987). Again, the production/modification of the capsule may be under the control of soluble factors found within the aqueous or vitreous humors which signal the lens cells to alter their extracellular matrix (ECM). Regardless of the cause of the differences found within the lens capsule, the function of the heterogeneity could be to direct or sustain cellular migration along the capsule, to provide morphogenetic cues which participate in fiber cell differentiation or to serve as sites for binding and presenting factors responsible for stimulating lens cell differentiation. The soluble proteins found in the aqueous or vitreous humors, as well as those produced by the differentiating cells themselves, could potentially regulate the differentiation of lens cells. Thus, both the state of the ECM and the presence of soluble factors play a primary role in the morphology and biochemical behavior of lens cells.

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine found in a large variety of cells and tissues. Lens epithelial and fiber cells plus both aqueous and vitreous humors contain TGF-β (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Potts, Bassnett and Beebe, 1995). TGF-β has the ability to bind heparan sulfate proteoglycans, fibronectin and type IV collagen, suggesting that it should also be a component of the lens capsule (Boyd, et al., 1990; Fava and McLure, 1987;

Paralkar, Vukicevic and Reddi, 1991; Attisano, et al., 1994). Several well studied functions of TGF-β include the regulation of cell proliferation and differentiation as well as the secretion of ECM proteins and their degrading proteinases (Sporn and Roberts, 1992; Attisano, et al., 1994). TGF-β, therefore, has the ability to affect cell functioning directly and indirectly by modifying the composition and structure of the matrix associated with the targeted cells. The specific responses elicited by TGF-β stimulation depend upon the targeted cell type, the cells' interaction with the surrounding ECM and the presence of other regulatory proteins. These observations seem to suggest that TGF-β in the lens could function in keeping lens cells withdrawn from the cell cycle and in affecting the composition of the lens capsule.

Following are brief accounts on 1) lens development, morphology and terminal differentiation; 2) TGF-β, TGF-β receptors and possible signaling cascades; 3) the enzymes capable of degrading ECM components and 4) the interactions of the enzymes, TGF-β and the ECM.

LENS DEVELOPMENT

The embryonic development of the vertebrate ocular lens is comprised of a series of transient morphological structures found at specific embryonic stages. Before presumptive lens cells begin to differentiate from the cranial surface ectoderm, the optic vesicles have already formed as lateral outgrowths of the diencephalon or forebrain. The optic vesicles have expanded through the surrounding mesenchyme and reached the surface ectoderm. Signals originating from the optic vesicle induce cells of the adjacent surface ectoderm to elongate into a thickened lens placode. The center of the lens placode invaginates into a lens pit which deepens, forming a cup-like structure. The rims of the lens cup approach each other and fuse, resulting in the

formation of a hollow, spherical lens vesicle. The lens vesicles pinch off from the surface ectoderm. Each vesicle is encased by the basement membrane originating from the surface ectoderm. Cells located in the posterior of the lens vesicle begin to elongate into the lumen. These elongating cells form the primary lens fibers, which ultimately obliterate the lumen of the lens vesicle (O'Rahilly and Meyer, 1959; McAvoy, 1980; Rafferty, 1985).

The obliteration of the lumen of the lens vesicle defines three areas within the developing lens: the anterior mitotic epithelium, a transitional region located at the equator and the posterior primary lens fibers. The anterior epithelium remains as a single layer of cuboidal cells, the lens epithelium. The equatorial region contains cuboidal epithelial cells which elongate into secondary lens fiber cells. The secondary lens fibers are then added as concentric shells to the periphery of the primary fibers. This establishes the basic pattern of morphology for the remainder of development and for the continuous growth of the lens during post-natal life (figure 1).

Around the time of birth, the anterior lens epithelium ceases to be mitotic. Proliferating epithelial cells become confined to a pre-equatorial ring called the germinative zone (Persons and Modak, 1970). Daughter cells of this zone migrate posteriorally along the lens capsule. Within the equatorial region, the post-mitotic daughter cells begin to elongate and differentiate into fiber cells. In birds and reptiles, the germinative zone lies more anterior to the lens equator than in other vertebrates. This creates an anterior region of post-mitotic elongating cells called the annular pad, located between the germinative zone and the equatorial fibers (Hanna and Keatts, 1966). As cells elongate, their axis of orientation rotates. The basal surface of the elongating fiber cell remains attached to the lens capsule while the apical membrane

opposes the apical membranes of the lens epithelium. As in the embryo, new fiber cells are added atop pre-existing cells. Due to the varied length of the newly formed fiber cells, their centrally located nuclei form the "bow region" of the lens (Kuwabara, 1975). The innermost cells of the bow region eliminate their nuclei and cytoplasmic organelles. The anterior ends of these fiber cells eventually form apico-apical junctions with cells originating from the opposite side of the lens. These junctions form the anterior sutures of the lens. The basal ends of the fiber cells remain in contact with the posterior lens capsule. Posterior lens sutures are formed well after the cell's apical surface has been inserted into the anterior suture. Therefore, the fiber mass of an adult lens can be subdivided into: newly formed superficial cortical fibers retaining their nuclei and basal attachment to the lens capsule, deep cortical fibers inserting into both lens sutures and the nuclear fibers consisting of the embryonic primary lens fiber cells.

LENS DIFFERENTIATION

Terminal differentiation of lens epithelial cells into lens fiber cells is a continuous process which involves a variety of morphological and biochemical changes. These changes include withdrawal from the cell cycle, cellular elongation, denucleation, DNA degradation and the elimination of cellular organelles. Differentiation also entails the alteration of cytoskeletal components plus the accumulation of lens specific crystallins and several membrane specializations.

CYTOPLASMIC CHANGES IN MATURING LENS CELLS

The post-mitotic progeny of cuboidal lens epithelial cells differentiate into greatly elongated lens fiber cells. Fiber cells located at the interior edge of the bow region lose their nuclei as a consequence of nuclear membrane fragmentation.

Concurrent DNA degradation results from the accumulation of single stranded DNA breaks. The nucleoplasm eventually becomes indistinguishable from the fiber cell's cytoplasm (Piatigorsky, 1981). Loss of mitochondria and endoplasmic reticulum coincide with the loss of nuclei. The golgi apparatus and other organelles are absent from the cytoplasm by the time the cells become deep cortical fibers. The cytoplasm of lens fiber cells appears as a fine granular substance due to the accumulation of crystallins (Kuwabara, 1975; Bassnett, 1995).

CHANGES IN LENS CRYSTALLIN SYNTHESIS

Crystallins comprise 90% of the total soluble protein found within a lens fiber cell. They are highly stable proteins which must retain their structural and functional integrity continuously throughout the life of the organism (Horowitz, 1993). There exists four immunologically distinct classes of crystallins and each class contains multiple polypeptides. Vertebrate lenses contain α-, β- and γ-crystallins. Avian and reptilian lenses contain δ-crystallins in the place of γ-crystallins. Crystallin synthesis differs markedly between the species. Yet, within a specific organism, the sequential appearance and regional localization of the different classes of crystallins are tightly regulated. Once expressed, the polypeptide composition of each class of crystallins also changes in a species-specific manner as lens fiber cells continue to mature. Transparency relies on the coordinated expression and organization of the many crystallin polypeptides found within lens cells (Piatigorsky, 1981; Van Leen, et al., 1987; Piatigorsky, 1989).

CHANGES IN LENS CELL CYTOSKELETON

Cytoskeletal changes occur within a lens fiber cell as differentiation and maturation continue. Microtubules, microfilaments and the intermediate filament,

vimentin, can be found in various amounts within the lens epithelium. The highest amounts of microtubules are contained within the cytoskeleton of fiber cells located in the deep bow region (Kuwabara, 1975). Vimentin is the most predominant cytoskeletal component of lens epithelial cells, but can be found in decreasing abundancy as fiber cells mature. Both microtubules and intermediate filaments are absent from the cytoskeleton of nuclear fiber cells. Lens epithelial cells contain less actin than lens fiber cells. Microfilaments are oriented along the long axis of the fiber cell. Actin can be found in close apposition to fiber cell membranes, although cytoplasmic staining is also observed (Ireland, 1982).

Beaded filaments are additional cytoskeletal structures found specifically in vertebrate lens fiber cells. A beaded filament is composed of a 5-7 nm diameter backbone, associated with 20 nm beads spaced at regular intervals along the backbone (Maisel and Perry, 1972; FitzGerald and Casselmann, 1991). Chick beaded filaments are mainly composed of two polypeptides: a protein with molecular weight of 49 kD, named phakinin and a 95 kD protein named filensin (Merdes, et al., 1991; Merdes, Gounari and Georgatos, 1993). Both of these proteins belong to the intermediate filament family of cytoskeletal proteins and show high sequence homology to acidic cytokeratins (Merdes, et al., 1991; Masaki and Watanabe, 1992; Remington, 1993). Phakinin and filensin are located throughout the cytoplasm of annular pad cells, constituting only minor components of their cytoskeleton. During the initial stages of lens fiber elongation, beaded filaments are located within the cytoplasm and in association with the plasma membrane. As differentiation proceeds, the amounts of both proteins increase dramatically and the locale of the beaded filaments is restricted to the plasma membrane of mature fibers (Maisel, et al., 1977; Ireland and Maisel,

1984; FitzGerald, 1988). It is not known how beaded filaments are assembled from phakinin and filensin or how these filaments become associated with the lens fiber membrane. Both proteins are synthesized as basic isoelectric variants. Both become rapidly acidified by a series of phosphorylations (Ireland and Maisel, 1989). The possibility exists that the membrane association of beaded filaments may result from extensive post-translational modification. *In vitro* experiments showed that cAMP-dependent phosphorylation of phakinin and filensin results in an increase in their membrane association (Ireland and Maisel, 1988; Ireland, Klettner and Nunlee, 1993).

CHANGES IN LENS CELL PLASMA MEMBRANES

MORPHOLOGY. As lens fibers elongate and differentiate, their associations with adjacent cells and the lens capsule changes. As fibers enter into the lens sutures, the cells lose associations with the lens epithelium and the posterior lens capsule and develop complex intercellular connections with adjacent cells. The lateral membranes of lens epithelial cells are markedly infolded and contain adherens junctions, desmosomes and gap junctions (Kuwabara, 1975; Lo, 1988). As epithelial cells differentiate into lens fibers, the area of plasma membrane increases over 1000 fold (Rafferty, 1985). Fibers become long flat hexagonal shaped cells, oriented with their broad faces running parallel to the lens surface. The lateral infoldings of lens epithelial cells become elaborated into lateral interdigitations between adjacent fiber cells. These lateral interdigitations are projections of lateral membrane located along the cells edges. The edges are formed by the intersection of broad and narrow or two narrow faces of the hexagonal fiber cell. The projections, known as 'balls', fit into corresponding depressions or 'sockets' found along the edges of the abutting fiber cell. Smaller balls and sockets are located between the broad faces of apposing fibers.

Balls and sockets act as interlocking devices to assure proper fiber cell alignment (Kuwabara, 1975; Kuszak, Bertram and Rae, 1986).

CELLULAR ADHESION MOLECULES. Differentiation is associated with a change from cell-cell and cell-substrate interactions to entirely cell-cell contacts. Both types of interactions depend upon the expression of cellular adhesion molecules. Integrins are the primary adhesion receptors for proteins found in the extracellular matrix, although integrin based cell-cell interactions have been observed (Larjava, et al., 1990; Hynes, 1992). Individual integrins are heterodimers of covalently linked α and β subunits. The specific combinations of the various subunits determines the specificity of interactions with particular extracellular matrix components. The β_1 subunits are found primarily in integrin receptors for basement membrane proteins. Within the chick lens, β_1 subunits have been localized to the basal membranes of epithelial and fiber cells in contact with the lens capsule (Menko and Philip, 1995). β_1 subunits were also found between epithelial cells, between fiber cells and between epithelial and fiber cells. Of the α subunits, α_3 and α_6 are the only ones detected, to date. The α_3 subunit binds fibronectin, laminin and collagens. It was located in membranes of both epithelial and fiber cells, with more expression in the epithelial cells. The α_6 subunit, which binds laminin, was primarily associated with fiber cells. Since integrins are linked to the cytoskeleton, differential expression of integrin subunits may influence different stages of differentiation.

Cell-cell interactions involve families of glycoproteins known as cell adhesion molecules (CAM) and cadherins. Classically, calcium-dependent cell-cell adhesion involved the cadherin family of proteins, while calcium-independent adhesion was performed by CAMs (Geiger and Ayalon, 1992). Distinctions between the two groups

are waning. Neural cadherins (N-cadherins) are also known as adherens junction specific CAMs or A-CAMs (Takeichi, 1988). N-cadherins have been detected in human, bovine and chick lenses. Chick lens epithelial and annular pad cells express N-cadherin at low levels. Levels of N-cadherin peak within the membranes of cortical fiber cells and decrease to markedly less N-cadherin in membranes of nuclear fibers (Heslip, et al., 1986). A highly polysialylated form of neural CAM or N-CAM has been localized to membranes of epithelial and annular pad cells of the chick lens (Watanabe, et al., 1992). Unlike N-cadherin, the expression of N-CAM declines markedly in cortical fibers and is at undetectable levels in lens nucleus. The levels of polysialylation also decrease as the lens cells mature.

LENS CELL COMMUNICATING JUNCTIONS. Lens epithelial cells are coupled to their neighbor cells by typical gap junctions (Kuwabara, 1975). Gap junctions are membrane specializations containing intercellular channels which allow inorganic ions, sugars, amino acids, nucleotides, vitamins and other small molecules to pass freely between communicating cells (Caspar, et al., 1977). Gap junctions appear as pentalamellar structures in electron microscope micrographs and as tightly packed clusters (or crystalline arrays) of intramembranous particles (IMP) when viewed in freeze-fracture studies (Larsen, 1977). Each IMP of a gap junction represents an individual connexon. Connexons are composed of six transmembrane proteins or connexins arranged cylindrically around a 1.5-2 nm pore. Connexons of adjacent cells align and form a continuous aqueous channel joining the two different cytoplasms.

Connexins belong to a multigene family. The expression of connexins is cell type specific. Connexin43 (Cx43) is located in the membranes of chick lens epithelial cells and superficial fiber cells of the bow region (Jiang, Paul and Goodenough, 1993).

Cx56 and Cx46.5 are strictly located in fiber cell membranes (Jiang, et al., 1994). Junctional membranes of fiber cells resemble gap junctions by containing connexins and displaying a pentalamellar structure (Lo and Reese, 1993; Michea, de la Fuente and Lagos, 1994). But, unlike gap junctions, fiber junctions exhibit a random, non-crystalline array of IMPs and a much narrower intercellular space. The intercellular space of fiber junctions measures approximately 0.5 nm. Whereas, membranes of typical gap junctions are separated by a 'gap' of 2-3 nm.

The membranes of chick fiber cells are 50-60% covered with fiber junctions (Kuszak, Maisel and Harding, 1978). Major intrinsic protein (MP26) is undetectable in lens epithelial cells, but constitutes 50-60% of the proteins isolated from lens fiber cell plasma membranes (Paul and Goodenough, 1983; Michea, de la Fuentes and Lagos, 1994). It was therefore assumed that MP26 was the putative junction protein of fiber cells. However, MP26 is not structurally related to the connexins. MP26 molecules form tetragonal, not hexagonal particles. MP26 can be localized to fiber junctions, but is found within only one of the apposing membranes and abuts only particle-free sections. MP26 contains three positively charged extracellular loops. It has been suggested that MP26 functions to promote cell-cell adhesion by electrostatic interactions between its extracellular loops and the negatively charged lipids within the opposing membrane (Michea, et al., 1995).

LENS CAPSULE

The ocular lens is encased entirely within the lens capsule, the thickest basement membrane found in the body (Haddad and Bennett, 1988). The lens capsule originates from the basement membrane of the presumptive lens ectoderm (Parmigiani and McAvoy, 1991). As the lens vesicle detaches from the cranial surface ectoderm,

the basement membrane surrounding the vesicle thickens by the deposition of layers of basement membrane material from the basal surface of the cells. This produces a lamellar appearing lens capsule. As thickening continues, lamellae become indistinguishable and the lens capsule appears as a homogeneous meshwork of tiny fibrils arranged in parallel to the lens surface (Kuwabara, 1975; Cammarata, et al., 1986; Reyer, Liou and Pinkstaff, 1994).

The lens capsule grows continuously throughout life (Young and Ocumpaugh, 1966). The surface area of the lens capsule increases to accommodate the ever expanding lens fiber mass. Lens capsules of embryonic chickens show an eleven fold increase in surface area between embryonic day six and day twenty (Johnson and Beebe, 1984). The lens capsule also continues to thicken, the anterior capsule becoming much thicker than the posterior (Kuwabara, 1975; Piatigorsky, 1981). The thickness of the lens capsule varies depending upon position along the capsule and with the particular age and species of the organism.

Both lens epithelial and fiber cells synthesize and secrete capsular components at their basal surface (Young and Ocumpaugh, 1966; Rafferty and Goossen, 1978; Fitch, Mayne and Linsenmayer, 1983; Laurent, et al., 1987). Autoradiographic studies have shown newly synthesized collagen as a distinct band that slowly migrates outward away from the lens surface. Other studies show, in addition to lamellar growth, the rapid appearance of a diffuse labeling pattern of an unidentified constituent that has a turnover rate of approximately one month (Haddad and Bennett, 1988).

The lens capsule is mostly composed of basement membrane specific collagen type IV (Cammarata and Spiro, 1985). Fibrillar collagens type I and type III have also been detected (Marshall, et al., 1992). Type IV collagen assembles into a

nonfibrillar mesh-like scaffold to which non-collagenous components of the lens capsule attach. This forms a complex, yet organized matrix. The non-collagenous component of the lens capsule is comprised of heparan sulfate proteoglycans and the glycoproteins laminin, entactin and fibronectin (Cammarata and Spiro, 1985). Heparan sulfate proteoglycans consist of a core glycoprotein covalently linked to heparan sulfate glycosaminoglycans (GAGs). Proteoglycans and their associated GAGs form hydrated gels of various charges and pore sizes, through which molecules may rapidly diffuse. Proteoglycans also bind and regulate the activities of secreted proteases, protease inhibitors and signaling molecules. Immobilization of such proteins can either inhibit or enhance the secreted molecules activity. Laminin is composed of three long polypeptide chains arranged as an asymmetrical cross. Laminin contains specific domains that bind type IV collagen, heparan sulfate, entactin and cell-surface laminin receptors. Entactin is a dumbbell-shaped molecule that binds to both laminin and type IV collagen. Fibronectin is a dimer of two large subunits joined near their carboxyl terminus by a pair of disulfide bonds. Specific domains within the fibronectin dimer bind collagen, heparin and cell-surface receptors (Inoue, 1989).

Immunocytochemical labeling revealed that entactin comprises a major portion of the non-collagenous components of the lens capsule. Heparan sulfate proteoglycan and laminin were found in lower abundance. Fibronectin was the least abundant of the capsular glycoproteins (Cammarata, et al., 1986). Labelling for both collagen and the non-collagenous components showed an even distribution of components throughout the thickness of the lens capsule. The even pattern of distribution reflects the co-localization of the various components and their natural association into a complex matrix (Cammarata, et al., 1986; Marshall, et al., 1992; Arita, et al., 1993). Contrary

to the results mentioned above, autoradiographic and histochemical data presented by Webster Jr., et al. (1987) demonstrated that sulfated materials within the lens capsule localized into distinct laminae. Lovicu and McAvoy (1993) using immunohistochemical staining of heparan sulfate proteoglycans also demonstrated reactivity in the form of laminae. The anterior capsule exhibited bands of reactivity as laminae localized along both the external and internal edges of the capsule. Equatorial capsule had one centrally located heparan sulfate proteoglycan reactive band, while the posterior capsule showed two narrow laminae within the capsule.

Although the lens capsule is a single continuous basement membrane, the individual constituents are differentially distributed between the anterior and posterior regions of the capsule. The concentration of collagen and laminin is higher in the anterior than the posterior capsule. Whereas, entactin, fibronectin, levels of carbohydrate and heparan sulfate proteoglycans are more abundant in the posterior capsule (Johnson and Beebe, 1984; Mohan and Spiro, 1986). Not only does heparan sulfate proteoglycan differ in concentration between anterior and posterior capsule, but different species preferentially exist. Heparan sulfate proteoglycans from the anterior capsule have a molecular weight around 400 kD. Those of the posterior capsule are larger, having a molecular weight exceeding 500 kD.

The heterogeneity in composition existing between the different regions of the lens capsule could be explained by several non-exclusive possibilities (Fitch, Mayne and Linsenmayer, 1983; Johnson and Beebe, 1984). First, heterogeneity could be the result of distinct cell types preferentially synthesizing different ECM constituents. Both epithelial and fiber cells synthesize capsular components (Young and Ocumpaugh, 1966; Rafferty and Goossen, 1978; Fitch, Mayne and Linsenmayer, 1983;

Laurent, et al., 1987). The possibility exists that the different cell types, upon receiving the same 'ECM producing' signals, produce different basement membrane components. Second, it is possible that the distinct cell types receive different 'ECM producing' signals due to their specific location within the lens. Third, enzymes preferentially found within the aqueous or vitreous humors may degrade particular ECM molecules within a homogeneously produced lens capsule. Fourth, the lens cells themselves, depending upon signals within the different external environments, may synthesize ECM degrading enzymes.

Fitch, Mayne and Linsenmayer (1983) state that basement membranes, which play an informative role, should be heterogeneous either in molecular composition or in the way their constituents assemble. The lens capsule is heterogeneous on both accounts. This is highly suggestive of specific regional functions of the lens capsule. As mentioned above, lens cells contact different matrices bathed in different ocular fluids, as they migrate along the lens capsule during terminal differentiation. Differences between aqueous and vitreous humors have been demonstrated by experiments in which the epithelium of inverted lens differentiated into lens fiber cells, presumably induced by factors within the vitreous humor (Coulombre and Coulombre, 1963). Recently, Lovicu, Chamberlain and McAvoy (1995) cultured rat lens epithelial explants in the presence of aqueous or vitreous humor. Only cells exposed to the vitreous humor underwent changes characteristic of lens fiber differentiation and produced an ECM. Therefore, the different factors contained within the vitreous humor seem to be modifying lens cell behavior.

The composition of the basement membrane influences cellular behavior (Cammarata and Spiro, 1985; Webster Jr., et al., 1987; Juliano and Haskill, 1993;

Mooney, Langer and Ingber, 1995). Individual ECM glycoproteins can directly alter cell behavior through signals created by their specific interactions with cell-surface integrins. Heparan sulfate proteoglycans, bound to the ECM molecules, serve as a selective molecular filter for factors within the ocular media. Heterogeneity in heparan sulfate proteoglycan concentration and species as well as the difference in thickness between the regions of the lens capsule suggests that these factors may or may not diffuse through the capsule to reach the lens cells. Heparan sulfate proteoglycans also binds growth factors, proteases and their inhibitors. Therefore, the lens capsule, like other basement membranes, could indirectly alter cell behavior by functioning to: 1) concentrate these factors; 2) to protect these factors from degradation; 3) to act as a reservoir for their delayed release; 4) to localize their area of activity or 5) to block their activity. The heterogeneity of the lens capsule, therefore suggests that lens cells do receive varying signals, depending upon their particular location within the lens. In summary, it seems that the physiological state of lens cells is influenced directly or indirectly by constituents of the capsule.

TRANSFORMING GROWTH FACTOR-BETA

Transforming growth factor beta (TGF-β) was named for its ability to stimulate anchorage-independent growth of normal fibroblasts (Roberts, et al., 1981). It was also isolated as tumor inducing factor (TIF-1) from glioblastoma cells and as cartilage inducing factors (CIF-A and CIF-B) from bovine bone (Iwata, et al., 1985; Seyedin, et al., 1985). It is now known that TGF-β influences a wide variety of cellular functions, such as the control of cell division during terminal differentiation and extracellular matrix production in a large variety of cell types. TGF-β is involved in both normal physiological processes like wound healing and morphogenesis as well as in

pathological states such as vitreoretinopathy, glomerulonephritis and cancer. This multifunctional protein may also elicit opposite responses within the same cell type, depending upon its concentration and the microenvironment in which the specific cell resides (Attisano, et al., 1994).

TGF-β serves as prototype for the TGF-β superfamily of signaling molecules. Most members of the superfamily are involved in growth regulatory functions. Based on primary structure and biological similarities, the members are arbitrarily divided into three groups (Attisano, et al., 1994). The largest and most diverse group is composed of the bone morphogenetic proteins (BMP), decapentaplegic proteins (dpp) and the Vg1 protein. These function in bone morphogenesis, *Drosophila* dorsoventral patterning and *Xenopus* inductive tissue interactions. The second group contains the activins, inhibins and Müllerian inhibiting substance (MIS). These proteins regulate hormonal secretion in the pituitary gland and the regression of the paramesonephric ducts in male embryos. The different isoforms of TGF-β (TGF-β1 through TGF-β5) make up the third group. TGF-β proteins exist as 25 kD homodimers, although the heterodimer TGF-β1.2 has been identified. The mammalian forms consist of only TGF-β1, β2 and β3. TGF-β4 and TGF-β5 are found in chickens and frogs, respectively (Sporn and Roberts, 1990).

TGF-β isoforms contain nine invariant cysteines. Seven of these are common among all members of the TGF-β superfamily (Daopin, et al., 1992; Schlunegger and Grütter, 1992). The TGF-β isoforms also share a high degree of amino acid sequence homology. There is approximately 70% homology between the isoforms β1, β2 and β3 and greater than 97% homology between species for each isoform (Massagué, 1990; Qian, et al., 1992). The degree of sequence conservation and the number of

isoforms maintained throughout evolution suggest the existence of specific important roles for each isoform; roles that still need to be determined.

Since TGF-β exerts a wide range of effects, its production and functions are under strict control. Transcription of TGF-β genes, like other genes, is regulated by specific response elements located within the promoter regions of the genes. The translation of TGF-β mRNA is regulated by the existence of an unusually long and GC-rich 5' untranslated region (Kim, et al., 1992). The GC-rich areas of the 5' untranslated region base-pair to form highly stable stem-loop structures. Stem-loop structures near the translational start site inhibit translation by preventing the 40S ribosomal unit from binding the mRNA. When translation occurs, the protein is synthesized in a latent form which needs to be activated after secretion in order to induce any biological effect (Lawrence, et al., 1984). Active TGF-β is then quickly cleared from the responsive cell by binding to ECM components or circulating binding proteins such as alpha-2 macroglobulin (Massagué, 1990).

Active TGF-β exists as a 25 kD dimer composed of two identical 112 amino acid peptides. TGF-β4 has 114 amino acids (Daopin, et al., 1992; Schlunegger and Grütter, 1992). The structure of the active TGF-β dimer has been determined by X-ray crystallography. The monomer subunit resembles a slightly bent left hand with only two fingers. Of the nine invariant cysteine residues, eight are involved in intrachain disulfide bonds. Three of the intrachain disulfide bonds form an unusual structure known as the TGF-β knot, located within the palm of the TGF-β monomer. Hydrophobic regions lie along the heel and finger tips. TGF-β molecules dimerize with the heel of one monomer abutted against the fingers of the companion subunit. A central cavity, formed between the two palms, is accessible to four water molecules.

The dimer is stabilized by a single interchain disulfide bridge between subunits at cysteine residues 77. Hydrophobic interactions between the subunits and extensive hydrogen bonding to the water molecules also contribute to the stabilization.

The individual TGF-β subunits are synthesized from one gene as a large latent precursor molecule of 390-412 amino acids (Derynk, et al., 1985; Olofsson, et al., 1992). The precursor molecule or the pre-propeptide contains the characteristic signal sequence of secreted proteins, causing the amino-terminus of the pre-propeptide to be cotranslationally cleaved at Gly29-Leu30 (Gentry, et al., 1988; Miyazono, et al., 1988). The propeptide is N-linked glycosylated at Asn82, Asn136 and Asn177. Proteolytical cleavage at Arg278-Arg279 separates the propeptide into two functional proteins: the mature TGF-β, consisting of the C-terminal 112 amino acids of the precursor molecule and the glycosylated N-terminal amino acids 30-278 known as the latency associated peptide (LAP). Both mature TGF-β and LAP exist as separate disulfide linked dimers which remain noncovalently associated (Wakefield, et al., 1988). Latency of mature TGF-β is dependent upon the association with LAP. It is thought that LAP masks the epitopes on TGF-β that are necessary for receptor binding (Lawrence, Pircher and Jullien, 1985). The LAP/TGF-β complex forms a structure known as small latent TGF-β (Olofsson, et al., 1992).

Small latent TGF-β can be found in association with a high molecular weight protein, forming a complex of 210-240 kD known as large latent TGF-β. The high molecular weight protein is a separate gene product from the TGF-β genes. The protein is a single peptide with the molecular weight of 125-160 kD in platelets and 170-190 kD in fibroblasts. Both are similar in structure and are not required for the maintenance of TGF-β latency. The best characterized high molecular weight protein

is known as latent TGF-β binding protein (LTBP). LTBP can be found in association with TGF-β1, β2 and β3. It is disulfide bonded to LAP without directly interacting with TGF-β. LTBP contains regions of homology with other polypeptides involved with protein-protein interactions. These common motifs include RGD sequences and EGF-like repeats. Therefore, it has been suggested that LTBP could participate in targeting TGF-β to the surface of cells or to the ECM. Recently, LTBP has been shown to function as a structural ECM protein, implying that latent TGF-β may be a component of the ECM. It is also thought that LTBP could function in the folding and secretion of latent TGF-β, since large latent TGF-β is secreted faster than small latent TGF-β (Miyazono, et al., 1988; Wakefield, et al., 1988; Okada, et al., 1989; Olofsson, et al., 1992; Taipale, et al., 1994; Dallas, et al., 1995).

TGF-β cannot bind to its receptors until mature TGF-β has released its associated LAP. Since almost all cells possess TGF-β receptors, the specific effect of TGF-β may be determined by the cell's ability to activate the latent molecule. Activation *in vitro* can occur with conditions of extreme pH (<4 or >9), presence of chaotropic agents, enzymatic alterations of the carbohydrates on LAP or by specific molecules such as the serine protease plasmin or an ECM glycoprotein, thrombospondin (Lyons, Keski-Oja and Moses, 1988; Schultz-Cherry, et al., 1994). Activation *in vivo* is still speculatory. It is thought that both plasmin and thrombospondin could be responsible for *in vivo* activation. Proteolytic activity of plasmin is located at the cell surface. Since both LAP and LTBP can bind cell surfaces, these components of latent TGF-β target the complexes to sites where activation by plasmin can occur. (Details of the plasminogen/plasmin system are described in the Matrix Metalloproteinase section.) Thrombospondin 1 activates latent

TGF-β by non-enzymatic means. Thrombospondin 1 contains two sequences that interact with latent TGF-β. The first sequence binds latent TGF-β and orients the molecule so the second sequence may interact with a site within the LAP dimer. Its thought that LAP then undergoes a conformational change which either releases active TGF-β or allows the complex to be recognize by cellular receptors (Schultz-Cherry, et al., 1995).

Once released from latency, TGF-β activity must be regulated in order to elicit the proper cellular response. Active TGF-β may bind to cellular TGF-β receptors and initiate signaling cascades within the specific cell. Active TGF-β may also be sequestered by betaglycan and other various ECM components. These molecules can serve to protect TGF-β from degradation, act as a reservoir for sustained release of the growth factor or to remove the molecule from the cell surface. Alpha-2 macroglobulin, a serum protein, inhibits TGF-β activity. It binds only active TGF-β because LAP blocks the alpha-2 macroglobulin recognition site on the TGF-β molecule. TGF-β complexed with alpha-2 macroglobulin can be cleared from circulation via the alpha-2 macroglobulin receptor (Fava and McLure, 1987; Wakefield, et al., 1988; Boyd, et al., 1990; Paralkar, Vukicevic and Reddi, 1991; Attisano, et al., 1994).

TGF-β RECEPTORS

At least nine different proteins have been identified as TGF-β receptors. Three bind TGF-β with affinities in the picomolar range and are ubiquitously expressed. Only two, TGF-β receptor type I and TGF-β receptor type II (TGF-β RI and TGF-β RII, respectively) actually function as signaling receptors. Betaglycan or TGF-β RIII may also be involved in TGF-β signal transduction, but only as a TGF-β presenting

protein. TGF-β RI and TGF-β RII are expressed at the cell surface at low levels (3,000 sites per cell) whereas betaglycan can number as many as 10^5 molecules per cell (Massagué, 1990; Attisano, et al., 1994).

Betaglycan exists as both a soluble and a membrane-bound form. The membrane-bound betaglycan is a 280 kD glycoprotein composed of a 110-130 kD core protein containing chondroitin and heparan sulfate glycosaminoglycans (GAGs). The structure of betaglycan, as determined by its amino acid sequence, suggests a long extracellular domain, a single transmembrane spanning region and a short cytoplasmic tail that lacks any signaling motif. Betaglycan becomes a soluble protein by cleavage of a site adjacent to the transmembrane region of the molecule. Both forms of betaglycan bind TGF-β1, β2 and β3 with equivalent affinities (Andres, et al., 1991; López-Cassillas, Wrana and Massagué, 1993).

TGF-β RI and TGF-β RII are glycoproteins similar in structure (Figure 2). Both receptors consist of a short extracellular domain containing conserved cysteine motifs, a single transmembrane spanning region and a cytoplasmic tail containing a serine/threonine (S/T) kinase domain. The amino acid sequence identity between different type I receptor kinase domains is more similar (60% homology) than the homology to TGF-β RII kinase domain (Lin, et al., 1992). TGF-β RII is a 75-110 kD glycoprotein with a longer extracellular domain and an extended (24 amino acid) serine/threonine-rich cytoplasmic tail (Massagué, 1992; Derynck, 1994; Franzén, Heldin and Miyazono, 1995). Type II receptors are constitutively phosphorylated, independent of the presence of TGF-β (Wrana, et al., 1994). They directly bind TGF-β1 and TGF-β3 with a much higher affinity than TGF-β2. Type II receptors also bind any TGF-β isoform complexed with betaglycan better than the free TGF-β dimer

(Derynck, 1994). The 53-70 kD TGF-β RI has a shorter extracellular domain and lacks the extended cytoplasmic tail of TGF-β RII (Massagué, 1992; Derynck, 1994). TGF-β RI does contain an additional cytoplasmic motif, the GS domain, located between the transmembrane region and the S/T kinase. This conserved 30 amino acid region contains the core sequence TTSGSGSG. The integrity of this domain is essential for TGF-β transmembrane signal transduction (Franzén, Heldin and Miyazono, 1995; Weiser, Wrana and Massagué, 1995). TGF-β RI does not directly bind TGF-β. It binds to the already formed TGF-β/RII complex (Wrana, et al., 1994).

TGF-β SIGNAL TRANSDUCTION

The TGF-β transmembrane signal transduction pathway is far from being completely understood. The binding of the active TGF-β dimer to TGF-β RII is independent of the presence of TGF-β RI. Yet TGF-β/RII cannot signal without TGF-β RI. The binding of TGF-β to TGF-β RI entirely depends on the previous formation of the TGF-β/RII complex. Betaglycan, when involved in TGF-β signaling, enhances binding of any TGF-β isoform to the type II receptor. Once TGF-β binds the type II receptor, TGF-β RI is recruited to form a stable trimeric complex. The constitutively active TGF-β RII transphosphorylates TGF-β RI within its GS domain. Activated TGF-β RI does not phosphorylate the type II receptor, but phosphorylates unknown downstream substrates. TGF-β RI has many isoforms. Each may initiate a different signaling cascade, resulting in the variety of cellular responses induced by TGF-β (Attisano, et al., 1994; Wrana, et al., 1994; Cárcamo, Zentella and Massagué, 1995; Chen and Weinberg, 1995).

The above model is stated in the simplest of terms. Heteromeric complexes of TGF-β RI and RII exist even in the absence of TGF-β (Chen, et al., 1995; Kawabata,

Chytil and Moses, 1995). TGF-β binding would then function to stabilize the pre-existing heteromeric complex and induce the transphosphorylation of the type I receptor. Homodimers of TGF-β RII have been found, suggesting the signaling receptor may be a multi-meric complex, most likely a tetramer. Recent data has shown that homologous complexes of TGF-β RII do not propagate TGF-β signals and that transphosphorylation of the TGF-β RI is essential. Therefore, TGF-β signal transduction must occur through a heteromeric receptor complex. The variety of TGF-β actions may be specifically regulated by the different receptor subtypes that have formed the functional multimeric receptor complex (Chen and Derynck, 1994; Henis, et al., 1994; Yamashita, et al., 1994).

The post-receptor signaling pathway(s) elicited by TGF-β stimulation are still incompletely known. Recently, Wang, et al. (1996) demonstrated that TGF-β RI associates with FNTA, the α-subunit of farnesyl transferase. Farnesyl transferase is involved in the activation of $p21^{ras}$. When the TGF-β/RII complex binds TGF-β RI, FNTA is released. FNTA complexes with its β-subunit to form the active enzyme which aides in the membrane association of $p21^{ras}$ and results in the activation of the RAS/MAPK signaling cascade. This data supports previous experiments by Mülder and Morris (1992) which showed that TGF-β stimulated the rapid activation of $p21^{ras}$ in epithelial cells whose growth is inhibited by TGF-β. Another possible signaling pathway in which TGF-β blocks the progression of the cell cycle involves the retinoblastoma protein (pRb). Underphosphorylated pRb is associated with the transcription factor E2F. The hyperphosphorylation of pRb by cyclin/cycle dependent kinase (cdk) complexes results in the release of E2F. E2F then initiates the subsequent transcription of genes required for the continuation of the cell cycle. It has been

shown that TGF-β prevents the formation of cyclin E-cdk2 and cyclin D-cdk4 complexes. Therefore, TGF-β stimulation keeps pRb underphosphorylated and prevents progression through the cell cycle (Laiho, et al., 1990; Geng and Weinberg, 1993; Slingerland, et al., 1994).

MATRIX METALLOPROTEINASES (MMPs)

The extracellular matrix (ECM) is composed of a specific set of proteins and proteoglycans intermingled into an intricate framework. Cells contacting the ECM not only receive structural support, but informational cues regulating basic cellular functions such as proliferation, differentiation, migration or adhesion. These cells, in turn, are responsible for the synthesis and degradation of the various ECM components. Thus, the ECM is not a static structure. ECM remodeling normally occurs during such physiological processes as development and growth, inflammation and wound-healing, ovulation and implantation plus the involution of the post-partum uterus and post-lactation mammary glands. Remodeling also occurs during pathological processes such as rheumatoid arthritis, tumor invasion and metastasis (Matrisian, 1990; Woessner Jr., 1991).

Agents affecting the ECM potentially affect normal and pathological cellular processes. Four classes of endopeptidases can degrade ECM molecules: aspartic-, cysteine- and serine-proteinases and the matrix metalloproteinases (MMPs). Each component of the ECM has a variable resistance against a specific degradative enzyme. A complex regulation of transcription, a multistep activation mechanism (at least in the case of plasmin and the MMPs) and the production of specific protease inhibitors function together to strictly control enzyme activity and insure the lack of aberrant proteolysis (Emonard and Grimaud, 1990; Woessner Jr., 1991).

Although MMPs are the focus of this review, serine proteinases need to be mentioned. Serine proteinases include enzymes such as plasmin, plasminogen activators, elastase and trypsin. This group can degrade denatured collagen and various glycoproteins found within the ECM. Serine proteinases also have the ability to activate MMPs, thereby indirectly enhancing matrix degradation. Once activated, MMPs can inactivate serine proteinase inhibitors. MMPs and serine proteinases potentiate the effects of each other to heighten the degree of matrix degradation (Emonard and Grimaud, 1990; Senior and Shapiro, 1992).

MMPs constitute the major proteolytic group responsible for both physiologic and pathologic ECM remodeling. Individual members degrade at least one ECM component, but as a group, MMPs can degrade the entire matrix. MMP gene expression is strictly regulated, but once transcribed the mRNAs are quickly translated and the proteins are rapidly secreted from the cell. All MMPs are secreted as soluble latent propeptides or zymogens, that require activation within the extracellular space to become biologically active. Activation *in vitro* can be caused by organomercurials, chaotropic agents or serine proteinases. Full enzymatic activity requires zinc at the active site, calcium for autocatalytic cleavage of the N-terminus and a neutral pH. MMP activity can be inhibited by chelating agents, specific tissue inhibitors of matrix metalloproteinases (TIMPs) or alpha-2 macroglobulin. Serine proteinase inhibitors have no affect on MMP activity (Matrisian, 1990; Senior and Shapiro, 1992; Mauviel, 1993).

MMP FAMILY

Based on substrate specificity, MMPs are arbitrarily divided into three classes. Collagenases (MMP1 and MMP8) denature fibrillar collagen types I, II and III to

gelatin. Gelatinases degrade denatured collagen types I, II and III; intact collagen types IV, V, VII and X; and elastin. Although the nomenclature for the gelatinases is confusing, this class has only two members. MMP2 is also known as gelatinase A, the 72 kD gelatinase or type IV collagenase. MMP9 is likewise known as gelatinase B, the 92 kD gelatinase or type V collagenase. Stromelysins 1, 2 and 3, matrilysin and metallo-elastase compose the last class. They can degrade proteoglycans, elastin, fibronectin, laminin, gelatin and the globular domains of type IV collagen (Matrisian, 1990; Woessner Jr., 1991; Mauviel, 1993).

The protein structure of MMPs is composed of several conserved domains arranged in the same linear order (Figure 3). MMP9, the largest matrix metalloproteinase, consists of six domains. The smallest MMP, matrilysin contains only the first three domains. These domains are essential for enzymatic activity. The N-terminus of all MMPs begins with a "pre" domain, composed of the characteristic signal sequence found in secreted proteins. The next 77 to 87 amino acids comprise the "pro" domain. This region contains a conserved cysteine residue located within the conserved sequence PRCGVPD. The cysteine interacts with the zinc at the active site of the enzyme, conferring latency upon the propeptide or zymogen. The "pro" domain is autocatalytically cleaved upon permanent activation of the enzyme. The last portion of matrilysin is the "catalytic" domain. This 162 to 173 residue long domain contains two conserved HEXGH sequences involved in binding zinc at the active site (Springman, et al., 1990; Murphy and Docherty, 1992).

All the rest of the MMPs contain a C-terminal "hemopexin-like" or "vitronectin-like" domain, 202 to 213 amino acids in length. This region of the protein is essential for the specific collagenolytic activity of collagenases. Both

gelatinases contain an extra "fibronectin-like" domain located within their catalytic domains. This area has sequence similarity to the collagen-binding domain of fibronectin and is thought to bind to denatured collagen, their substrate. MMP9 is the only matrix metalloproteinase to contain a "collagen-like" domain located between the catalytic and hemopexin domains. It has sequence homology to type V collagen and serves an unknown function (Matrisian, 1992; Murphy and Docherty, 1992).

MMP REGULATION

MMP expression can be induced in specific cell types by growth factors, cytokines and tumor promoters. The promoter region of the stromelysin and interstitial collagenase (MMP1) genes have been studied intensively. Less is known about the promoter regions of MMP9 and MMP2. The promoter region of MMP9 contains a TATA box and AP-1 site, whereas the MMP2 promoter region lacks both motifs. The MMP2 promoter region contains a cAMP response element and multiple SP1 sites. Although the promoter regions of MMP9 and MMP2 differ, TGF-β induces increased levels of both MMPs. Many MMPs seem to be coordinately expressed, yet differences in their promoter regions suggest diverse pathways which lead to the selective transcription of certain enzymes (Matrisian, 1992; Mauviel, 1993).

Once the MMP mRNA is expressed, translation and secretion of the zymogen occur rapidly. Controlled enzymatic activity depends on a multi-step activation process. The model of matrix metalloproteinase activation is called the cysteine switch mechanism. The zymogen remains latent due to the conformation of the molecule. The propeptide is folded back over the active site in such a way that the conserved cysteine within the sequence PRCGVPD of the "pro" domain complexes with a zinc molecule located at the active site. Activators of MMPs include reagents such as

aminophenylmercurial acetate (APMA) or sodium dodecyl sulfate (SDS) which interfere with the cysteine-zinc interactions or directly cause the propeptide to unfold. Alternatively, enzymes such as plasmin or trypsin cleave the propeptide upstream from the conserved cysteine. The proteolytic cleavage destabilizes the cysteine-zinc interactions, also allowing the zymogen to unfold. The unfolded zymogen forms an active intermediate which can autolytically cleave the remaining portion of the propeptide resulting in the permanently activated metalloproteinase. Permanent activation is always associated with the loss of approximately 10 kD of molecular weight from the N-terminus. The physiological activation of all MMPs, except MMP2, has been attributed to plasmin. MMP2 lacks a site for proteolytic cleavage. Studies have suggested that activation of MMP2 occurs by a mechanism which is associated with cell surfaces (Springman, et al., 1990; Woessner Jr., 1991; Murphy and Docherty, 1992).

The recent discovery of another class of MMPs have revealed the activation mechanism of MMP2. The new MMPs, called membrane-type matrix metalloproteinases (MT-MMPs), contain a transmembrane domain near their C-terminus. MT-MMPs are not secreted into the extracellular space, but remain inserted in the cell membrane. An activated MT-MMP acts as a cell surface receptor for one of the tissue inhibitors of metalloproteinases, TIMP-2. The TIMP-2 protein belongs to a family of inhibitors which specifically bind metalloproteinases. The MT-MMP/TIMP-2 complex then acts as a receptor for soluble proMMP2. The C-terminal domain of TIMP-2 binds the C-terminus of proMMP2. The formation of this tri-molecular complex is required for the activation of proMMP2 to MMP2. Still unresolved is the mechanism of activation of the MT-MMPs. Since they belong to the

MMP family, they should be inserted into the cell membrane as zymogens. It has been suggested that plasmin may also activate this class of MMPs. All MMPs that are activated by plasmin contain the amino acid sequence Arg-Arg-Lys-Arg. This sequence is found within the MT-MMPs. So, although plasmin does not directly cleave MMP2, it may still be necessary as the initiating step in the construction of the required tri-molecular activation complex (Sato, et al., 1994; Emmert-Buck, et al., 1995; Strongin, et al., 1995).

The activity of MMPs is regulated by alpha-2 macroglobulin and the family of TIMPs. Alpha-2 macroglobulin binds to active MMPs. A conformational change then occurs within the alpha-2 macroglobulin molecule, covalently entrapping the MMP and resulting in inactivation of the enzyme. The TIMP family of proteins contains three members that range in molecular weight from 20 to 30 kD (Hayakawa, 1994). All three TIMPs contain 12 conserved cysteine residues involved in six intrachain disulfide bonds, conferring stability against low pH and high temperatures. TIMP-1 and TIMP-2 share 40% amino acid sequence homology. TIMP-3 is approximately 40% homologous to TIMP-2 and about 30% homologous to TIMP-1. TIMPs noncovalently bind all active MMPs in 1:1 stoichiometry and cause the inactivation of their proteolytic activity. TIMP-1 can also noncovalently bind proMMP9 and TIMP-2 can bind proMMP2. The binding of a TIMP to a specific proMMP inhibits the autocatalysis of the propeptide. The TIMP/proMMP complex is still able to bind and inhibit an active MMP (Woessner Jr., 1991; Hayakawa, 1994).

PLASMINOGEN

Plasmin is involved in the activation of MMPs and latent TGF-β as well as the degradation of ECM components. Plasmin's broad range of activity require that its

production and enzymatic functioning be tightly regulated. Plasminogen activators (PAs), plasminogen activator inhibitors (PAIs), plasmin inhibitors and the involvement of cell surfaces all participate in controlling plasmin activity. Plasmin is produced from its zymogen, plasminogen (Pg), by the proteolytic cleavage of a single peptide bond catalyzed by the PAs. PAs exist in two forms, transcribed from two separate genes: tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA is secreted as an active 70 kD enzyme, while uPA is secreted as pro-uPA. Pro-uPA is activated by plasmin yielding uPA, a 40-55 kD enzyme. tPA and uPA can be found in soluble form or bound to cell surfaces via specific receptors. PAIs, located in the extracellular space, inhibit soluble uPA and tPA. Thus, most PA activity is associated with cell membranes (Vassalli, Sappino and Belin, 1991; Plow, et al., 1995).

PAs preferred substrate is Pg. Plasmin and Pg bind to the cell surface via their lysine binding sites (LBS) located adjacent to their proteolytic domain. LBS associate Pg and plasmin to the cell surface by recognizing and interacting with C-terminal lysines of membrane proteins. Because both zymogen and its activators are bound to the cell surface, the activation of Pg to plasmin is more efficient than that of soluble plasminogen (Plow, et al., 1986).

Once formed, plasmin may disassociate from the cell surface. Soluble plasmin is more rapidly inactivated by alpha 2-antiplasmin than cell-bound plasmin. This mechanism limits plasmin activity to near the cell surface. Plasmin remaining bound to the cell surface is involved in positive feed-back loops that enhances additional Pg activation. Cell-bound plasmin can activate more proPAs, leading to heightened PA activity. Plasmin bound to the cell surface can proteolytically alter membrane

proteins, thereby exposing more C-terminal lysines and recruiting additional Pg to the cell surface. These feed-back loops function to insure a sufficient amount of plasmin is always active. These complicated processes allow the cell to express a limited, yet affective proteolytic mechanism to degrade ECM, even in the presence of inhibitors for both PA and plasmin (Plow, et al., 1986; Vassalli, Sappino and Belin, 1991; Plow, et el., 1995).

INTERACTIONS BETWEEN TGF-β, MMPs, PLASMIN AND THE ECM

The dynamic nature of the ECM is maintained by complex interactions involving multiple mechanisms (Figure 4). The cells which produce and modify the ECM reciprocally receive specific cues from the ECM which affect cellular processes such as migration and differentiation. Plasmin degrades a broad spectrum of ECM components. Its association with the cell surface implies possible roles for plasmin in the migration of cells or in the localized release of matrix-bound growth factors which signal differentiation. Plasmin can also function to activate MMPs. Both classes of enzymes selectively degrade specific constituents of the ECM resulting in a modified ECM. MMPs may enhance the activity of plasmin by inactivating serine protease inhibitors like PAI. Therefore, MMPs and plasmin function in concert to degrade or modify the ECM.

Plasmin can also affect the composition of the ECM by activating TGF-β. Once activated, TGF-β can stimulate the synthesis of ECM constituents. TGF-β also regulates the production of proMMPs and PAs as well as their inhibitors, TIMPs and PAIs. The physiological state of a cell responding to TGF-β determines whether ECM components are synthesized or degraded. TGF-β stimulation also regulates the expression of integrin subunits (Ignotz and Massagué, 1987; Heino, et al., 1989). The

specific combination of the various subunits determines the specificity of the interactions between the cell and a particular component of the ECM. Therefore, TGF-β may control migration and differentiation directly, by modulating the ability of the cells to bind to the different ECM components and by regulating the production/modification of the ECM, or indirectly, by releasing matrix-bound growth factors as the ECM is modified.

RESEARCH OBJECTIVES

TGF-β has the ability to bind heparan sulfate proteoglycans, fibronectin and type IV collagen, suggesting that it could also be a component of the lens capsule (Fava and McLure, 1987; Boyd, et al., 1990; Paralkar, Vukicevic and Reddi, 1991; Attisano, et al., 1994). Lens epithelial and fiber cells plus both aqueous and vitreous humors contain TGF-β (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Potts, Bassnett and Beebe, 1995). It has been suggested that a function of TGF-β in the lens is the production of ECM components during capsule formation (Liu, et al., 1994). Additionally, TGF-β induced production of matrix degrading proteases may result in the differences observed between the various regions of the lens capsule. This implies that TGF-β, through its effects on ECM production and modification, may be a potential regulator of lens migration and differentiation. To date, this function of TGF-β in the lens has not been examined.

In support of the idea that TGF-β may affect lens cell migration and differentiation are studies examining the localization of uPA, an important component of TGF-β's matrix modifying activity (Tripathi, Tripathi and Park, 1990). Using immunocytochemistry, uPA was identified in the posterior lens capsule and in the equatorial epithelial cells, but could not be detected in the anterior capsule nor the

central epithelium. This distribution of uPA reflects functional differences in cellular motility between the different regions of the lens. Central epithelial cells are stationary and remain attached to the anterior lens capsule, therefore, uPA would not be expected to be expressed. Localization of uPA to the posterior capsule and equatorial epithelial cells suggests that these cells may be using the plasmin and MMP systems to produce a matrix capable of directing their migration. Since uPA synthesis is often controlled by TGF-β stimulation, this suggests that one possible function of TGF-β in the lens is establishing the highly localized expression of uPA.

Therefore, these studies were designed to: 1) identify TGF-β type I and type II receptors in chicken lens cells; 2) investigate the role of TGF-β in the mitotic withdrawal of differentiating lens cells; 3) investigate the role of TGF-β in the production of components of the lens capsule; 4) investigate the role of TGF-β in the production of enzymes capable of modifying the lens capsule.

AVIAN LENS

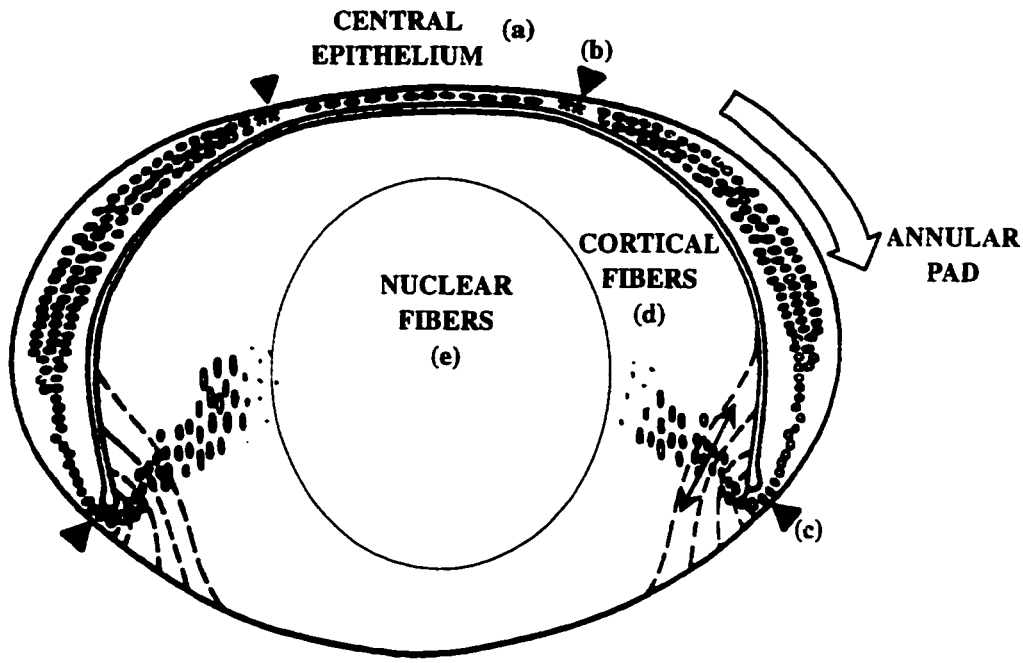


Figure 1. Schematic representation of an avian lens. (a) Post-mitotic central epithelium covers the anterior pole of the lens. (b) Germinative zone contains the mitotic stem cell population (**) of adult lenses. Post-mitotic daughter cells of the germinative zone form the annular pad as they migrate (in the direction of the open arrow) towards the lens equator. (c) The transitional zone consists of cells that are rotating their axis of orientation as they enter the fiber mass. (d) Cortical lens fiber cells elongate both anteriorly and posteriorly (double headed arrow) and begin to lose their cellular organelles. (e) Terminally differentiated nuclear fibers are surrounded by successively younger layers of cortical fibers. Illustration adapted from the work of Dr. Peggy Zelenka.

TGF-β RECEPTORS

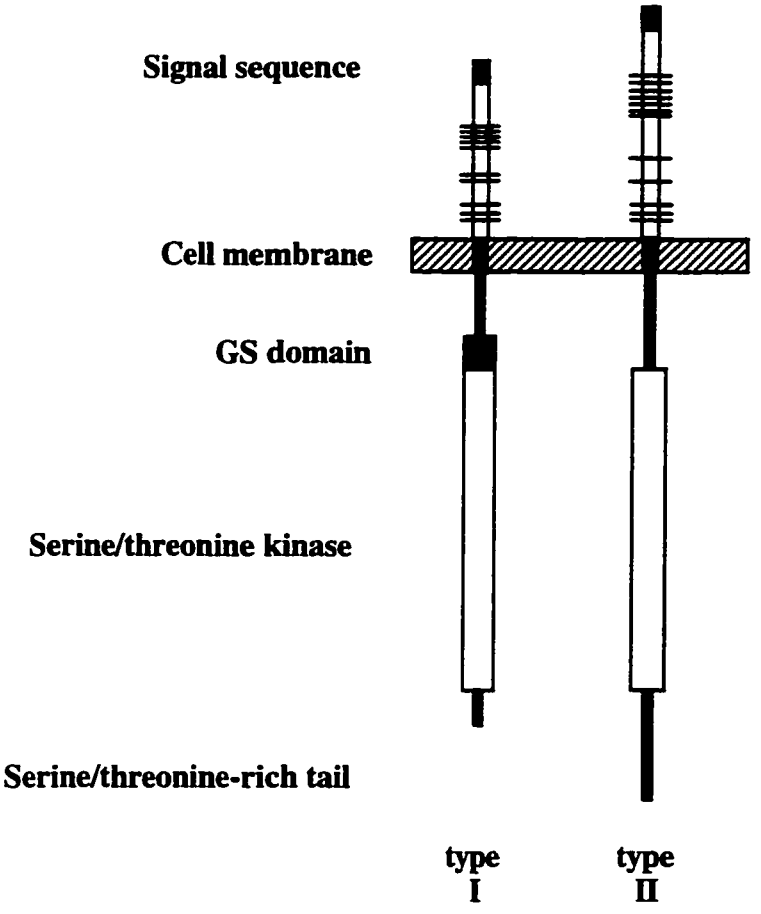


Figure 2. Schematic representation of TGF-β type I and type II receptors. Horizontal lines mark the locations of extracellular cysteine residues. The conserved TTSGSGSG sequence characteristic of type I receptors is located within the GS domain.

DOMAIN STRUCTURE OF MMPs

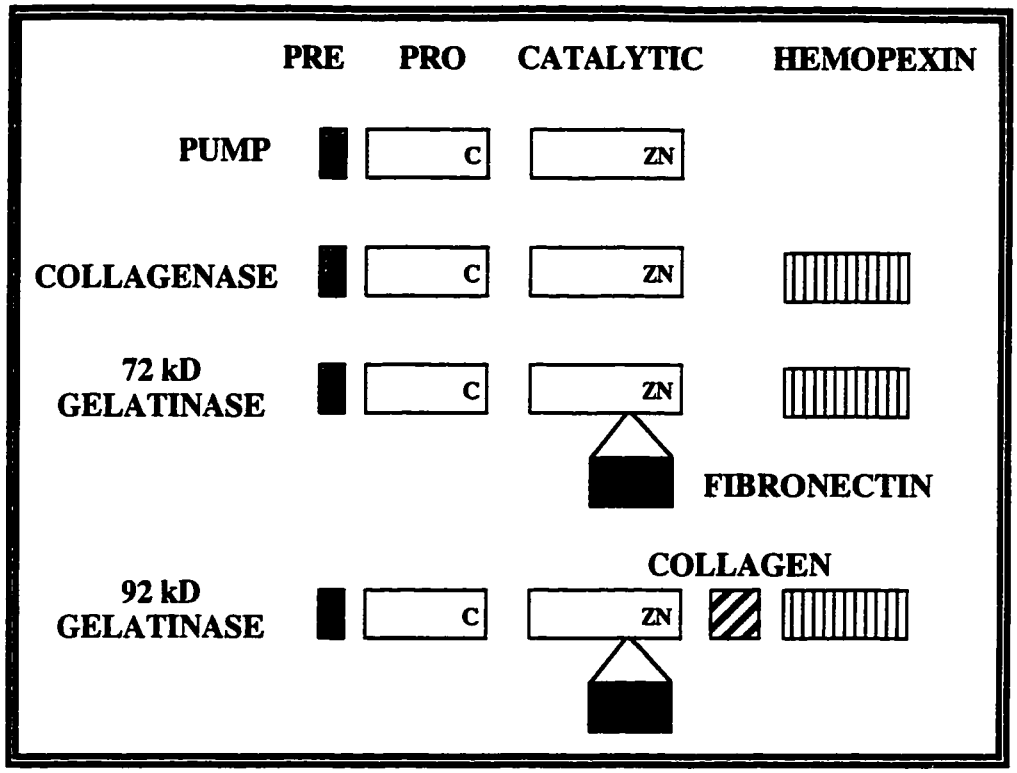


Figure 3. Schematic representation of the domain structure of MMPs. Boxes represent regions of amino acid similarities between the various MMP members. The PRE domain represents the signal sequence of the protein. The PRO domain contains the highly conserved PRCGVPD sequence (C). The CATALYTIC domain contains the active site with a bound zinc molecule (ZN). HEMOPEXIN domain found in all MMPs except PUMP. The FIBRONECTIN domain lies within the catalytic domain of only the gelatinases. It has sequence homology to the collagen-binding domain of fibronectin. Only the 92 kD gelatinase has an additional COLLAGEN domain, which has sequence similarities to type V collagen.

THE INTERACTIONS BETWEEN TGF-β, MMPs, PLASMIN AND THE ECM

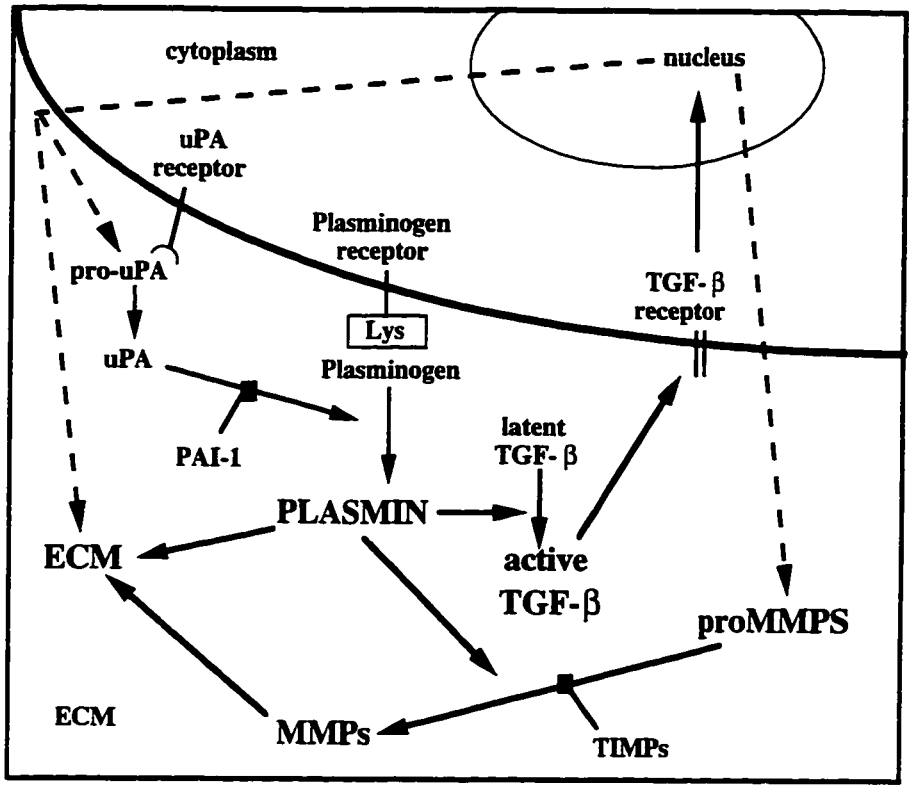


Figure 4. Schematic representation of the interactions between TGF-β, MMPs, plasmin and the ECM. Plasminogen system: Cell-bound plasminogen is activated to plasmin by receptor-bound PAs. Plasmin activates latent TGF-β and proMMPs and degrades a wide range of ECM components. Not shown in the diagram is α2-antiplasmin which inactivates free plasmin more rapidly than the cell-bound enzyme. TGF-β system: Latent and active TGF-β can bind ECM constituents. Active TGF-β binds TGF-β receptors and initiates still unknown intracellular signaling cascades. TGF-β stimulates the synthesis of ECM components and regulates the secretion of proMMPs and proPAs. TIMPs and PAI-1 inhibit the activity of MMPs and PAs, respectively. Not shown in the diagram is the TGF-β regulated production of latent TGF-β, TIMPs and PAI-1.

Chapter 2.

The Effects of TGF-β on the Proliferation and Differentiation of Cultured CLAP Cells.

INTRODUCTION

Along the periphery of the anterior epithelium of the ocular lens, cells destined to become lens fiber cells undergo their last mitotic division and begin the process of terminal differentiation. These cells migrate along the lens capsule towards the lens equator. Once beyond the equator, the cells rotate their apical-basal axis 180° and elongate into maturing lens fiber cells. The new fiber cells are packed as concentric shells atop existing lens fiber cells. Throughout terminal differentiation, the cells remain withdrawn from the cell cycle; synthesize increased amounts of lens-specific crystallins, cytoskeletal and membrane proteins; and become tightly associated with adjacent differentiating fiber cells (Persons and Modak, 1970; Kuwabara, 1975).

Fiber cell terminal differentiation may be under the control of genetic mechanisms or factors located in the microenvironments which encompass the lens cells. As lens cells migrate along the lens capsule, they encounter regional differences in the composition of the matrix (Kuwabara, 1975; Johnson and Beebe, 1984; Mohan and Spiro, 1986; Webster Jr., et al., 1987). These variations could function to direct or sustain migration, provide morphogenetic cues which participate in fiber cell differentiation or to serve as sites for the binding and presenting factors responsible for stimulating lens cell differentiation. During their migration, lens cells also encounter different ocular solutions. The anterior of the lens is bathed in aqueous humor, the equatorial region apposes the ciliary body and the posterior of the lens contacts the vitreous humor. Secreted proteins found in the different regions as well as those produced by the differentiating cells themselves, potentially could regulate lens cell differentiation (Coulombre and Coulombre, 1963; Lovicu, Chamberlain and McAvoy,

1995).

Transforming growth factor β (TGF-β) is a multifunctional cytokine found in a large variety of cells and tissues. Among the array of cellular responses elicited by TGF-β stimulation is the inhibition of epithelial cell proliferation and the regulation of cellular differentiation. One of the proposed pathways for TGF-β induced inhibition of growth stems from the observation that TGF-β treatment results in the underphosphorylation of the retinoblastoma protein (Laiho, et al., 1990). In mitotically active cells, cyclin/cyclin-dependent kinase (cdk) complexes phosphorylate retinoblastoma protein (pRb) which is associated with the transcription factor, E2F. Hyperphosphorylation of pRb triggers the release of E2F, which then initiates the subsequent transcription of genes, like *c-myc*, that are required for the continuation of the cell cycle. Data suggests that TGF-β prevents the formation of cyclin/cdk complexes, thereby keeping pRb underphosphorylated and in association with E2F, which consequentially blocks the cell cycle (Geng and Weinberg, 1993; Slingerland, et al., 1994). Fromm and Overbeek (1996) report that differentiating lens fiber cells cease expressing cyclins and cdks and that pRB is essential for maintaining the post-mitotic state of these cells. Thus, TGF-β within the lenticular environment may function to keep lens fiber cells in a post-mitotic state by blocking the expression of cyclin/cdk complexes.

TGF-β functions to alter cellular responses through signals transduced into the cell's interior by a heteromeric complex of two cell surface receptors. Both TGF-β type I and TGF-β type II receptors need to be functional in order for TGF-β to produce an intracellular signal (Derynck, 1994). Since TGF-β has been found in the

ocular environment as well as within lens cells (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Potts, Bassnett and Beebe, 1995), we wanted to test the hypothesis that TGF-β may influence the terminal differentiation of lens fiber cells by keeping them withdrawn from the cell cycle or by promoting the appearance of differentiated characteristics.

The model we have developed to examine mechanisms influencing fiber differentiation involves the cell culture of post-mitotic lens epithelial cells committed to fiber formation. These cells are obtained from an epithelial specialization found in chicken lenses known as the annular pad (Hanna and Keats, 1966). The annular pad is composed entirely of cells having undergone their final mitotic division. Any culture treatment is considered to have a positive effect on lens fiber differentiation if it produces previously observed characteristics of normal fiber cell formation.

In this report, we identify receptors for TGF-β on annular pad cells, with the use of specific antibodies, and characterize some of the *in vitro* responses of these cells in primary culture. Both type I and type II TGF-β receptors were identified in freshly isolated and primary cultures of annular pad cells. However, TGF-β application to cultured annular pad cells invariably resulted in the stimulation of cell division and a net negative effect on differentiated characteristics. Since annular pad cells are post-mitotic cells committed to terminal differentiation, our results indicated that the *in vivo* effect(s) of TGF-β must be integrated into the larger framework of receptor-mediated processes known to affect lens fiber formation.

MATERIALS and METHODS

Reagents. Unless otherwise noted, all reagents were obtained from Sigma

Chemical Company (St. Louis, MO, U.S.A.). Tissue culture media and supplements were purchased from GIBCO BRL (Grand Island, NY, U.S.A.) or Celox (Hopkins, MN, U.S.A.). Tissue culture plasticware was purchased from Corning Glass Works (Corning, NY, U.S.A.). Growth factors and collagen type IV were purchased from Becton/Dickerson Labware (Bedford, MA, U.S.A.). TGF-β2 was purchased from Genzyme Corporation (Cambridge, MA, U.S.A.). Rabbit polyclonal antibodies directed against TGF-β receptor type I (aa 158-179) and TGF-β receptor type II (aa 246-266) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Nitrocellulose membrane and BCIP/NBT color developing reagents were obtained from BIO-RAD Laboratories (Hercules, CA, U.S.A.). ECL Western Blotting System was purchase from Amersham International plc (Little Chalfont, Buckinghamshire, England). WXR-B X-ray film was purchased from Wolverine X-Ray Sales and Service Inc. (Detroit, MI, U.S.A.). Tritiated thymidine and ^{35}S-methionine were purchased from ICN Radiochemicals (Costa Mesa, CA, U.S.A.).

Cell Isolation and Culture. Chicken lens annular (CLAP) cells were isolated from 2- to 3-month-old chickens as previously described (Ireland, Tran and Mrock, 1993) with minor modifications. In some experiments, 300 μg of total protein were loaded into each well of 24-well tissue culture dishes. Wells contained 1 ml Medium-199 (M199) supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and 0.1% fungizone. Culture media did not contain serum, except where mentioned. To assure monolayer growth in the experiments performed to detect TGF-β receptors, prior to the addition of cells, tissue culture plates were coated with collagen type IV as previously described (Ireland, Braunsteiner and Mrock, 1993). Briefly, collagen type

IV was suspended in sterile PBS, added to the wells of 24-well culture dishes and allowed to incubate overnight at 37°C, yielding a final coating concentration of 1 $\mu g/cm^2$. The solutions were aspirated and the wells washed several times in culture media before the addition of cells. Cells were viewed and photographed with phase contrast optics.

Culture Treatments. Cells were treated immediately after being plated and every other day thereafter. Concentrated stocks of the growth factors were added directly to the culture medium to yield the indicated dosages. Each dosage was examined 4-6 times per experiment. Each experiment was repeated at least three times. Controls received no treatments.

Thymidine Incorporation, DNA and Protein Determination. At the indicated times, the cultured cells were processed for tritiated thymidine incorporation and DNA accumulation. Procedures for both tritiated thymidine incorporation and DNA determination have been previously published by Ireland, Tran and Mrock (1993). The determination of protein levels of cell lysates dissolved in sample buffer was performed following the protocol of Henkel and Bieger (1994). Five microliters of sample were dotted onto a piece of nitrocellulose membrane. Membrane was stained (10% acetic acid, 45% methanol, 0.1% amido black) and destained (90% methanol, 2% acetic acid) prior to elution of the protein dots in 50% ethanol, 50% NaOH (50 mM), 1 mM EDTA. Eluent solution was read by spectrophotometry at 630 nm using bovine serum albumin as a standard.

Metabollic Labeling with ^{35}S-Methionine. Metabollic labeling of cultured CLAP cells was done according to previously published procedures (Ireland, Tran and

Mrock, 1993). Briefly, cells were methionine-starved for 4 hours prior to an overnight exposure to ^{35}S-methionine in the presence or absence of the indicated concentrations of TGF-β. Cells were dissolved directly into sample buffer. Equal numbers of radioactive counts, as determined by liquid scintillation counting, were separated by SDS-PAGE and processed for autoradiography.

Gel Electrophoresis, Autoradiography and Western Blotting. Samples of whole cell lysates, equalized for total protein or numbers of radioactive counts, were subjected to sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels (Laemmli, 1970). Gels were stained, dried and exposed to Wolverine WXR-B X-ray film. Autoradiograms were developed after 7-10 days.

For Western blotting, the separated proteins were electrophoretically transferred from the polyacrylamide gels to nitrocellulose paper. Blots were probed with a previously characterized monospecific polyclonal antisera directed against phakinin, a 49 kD cytoskeletal beaded filament protein greatly enriched in lens fiber cells (Ireland and Maisel, 1984). Secondary antibodies were conjugated to alkaline phosphatase.

TGF-β Receptor Detection by ECL Western Blotting. Freshly isolated CLAP and superficial fiber cells as well as cultured CLAP cells, were individually dissolved in sample buffer. Samples, equalized for total protein, were subjected to SDS-PAGE and electrophoretic transfer, as described above. The nitrocellulose paper was probed with commercially available polyclonal antibodies directed against TGF-β type I (amino acids 158-179) or type II (amino acids 246-266) receptors. Secondary antibodies were conjugated to horseradish peroxidase and the enhanced chemiluminescence reaction was detected by exposure of the blot to Wolverine WXR-

B X-ray film.

Statistical Analysis. Two-factor analysis of variance (ANOVA) was used for statistical analysis of data. Because there was significant interaction between factors, individual groups were analyzed by one-factor ANOVA to find the simple effects. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

Identification of TGF-β Receptors. TGF-β receptors are found in almost every cell type. It was of interest to determine if these receptors were detectable in CLAP and lens fiber cells. CLAP cells were isolated as described above. Superficial fiber cells were obtained by gently peeling away the outer most layer of the fiber mass. Figure 5 shows the SDS-PAGE gel (panel A) and autoradiograms of the corresponding ECL blots probed with antibodies directed against TGF-β type I receptors (panel B) or TGF-β type II receptors (panel C).

A single band of approximately 63 kD, corresponding to TGF-β type I receptor, and a single band of approximately 95 kD, corresponding to TGF-β type II receptor, can be detected in freshly isolated CLAP cells (lanes 1). Both receptor types are also detectable in CLAP cells cultured as aggregates (lanes 3) or as monolayers (lanes 4). In our hands, TGF-β type I and type II receptors cannot be detected in freshly isolated superficial fiber cells (lanes 2).

Effects of Various Growth Factor Stimulation on Thymidine Incorporation. Multiple growth factors are known to influence diverse aspects of lens growth and differentiation. Part of this study was to determine if TGF-β stimulation could inhibit the mitosis of the lens cell population committed to fiber cell formation. The effects

of TGF-β were compared to those of other distinct classes of growth factors found in the eye. In 24-well plates, CLAP cells were cultured in serum-free M199 in the presence or absence of IGF-1, PDGF-AB, bFGF or TGF-β over the indicated range of concentrations. Figure 6 shows that cultured CLAP cells can be stimulated to synthesize DNA by all the various growth factors tested. Significant increases in tritiated thymidine incorporation into DNA were observed beginning at dosages of 10 ng/ml for bFGF, 1 ng/ml for PDGF-AB, 100 pg/ml for IGF-1 and 10 pg/ml for TGF-β. The relative potency of the various growth factors on thymidine incorporation was, in decreasing order, TGF-β, IGF-1, PDGF-AB and bFGF.

Effects of TGF-β Stimulation on CLAP Cell Division and Morphology. The above data demonstrated that TGF-β stimulated a dose-dependent increase in thymidine incorporation of CLAP cells cultured in serum-free media. The presence of TGF-β in serum-free media also stimulates a dose-dependent increase in the degree of cellular attachment and spreading. Characteristic spreading of CLAP cells cultured for 5 days in the absence or presence of the indicated concentration of TGF-β can be seen in figure 7a. In the absence of TGF-β, serum-free culture conditions stimulate only minimal amounts of CLAP cell division. The possibility exists that TGF-β functions to inhibit the mitosis of actively dividing cells. The inclusion of 20% serum in the culture media stimulates CLAP cells to re-enter the cell cycle and to exhibit growth in monolayers (Ireland, Tran and Mrock, 1993). Therefore, we examined the effects of TGF-β stimulation of CLAP cells cultured in the presence of 20% chicken serum. TGF-β stimulation of CLAP cells cultured in media containing 20% chicken serum induced a dose-dependent increase in the degree of cellular attachment and spreading

at a much accelerated rate. Characteristic spreading of cells cultured for 2 days in M199 supplemented with serum, in the presence or absence of the indicated concentrations of TGF-β, can be seen in figure 7b.

The tritiated thymidine incorporation of cultured CLAP cells from experiments similar to those seen in figure 7 are shown in Table 1. TGF-β stimulated dose-dependent increases in DNA accumulation and thymidine incorporation in CLAP cells cultured in both serum-free and serum-containing conditions. The specific activity of CLAP cells cultured in the absence of serum is higher than that of cells cultured in the presence of serum.

Time Response to TGF-β Isoforms. Studies were undertaken to examine the time course of thymidine incorporation, and the possible differences in mitotic activity, of CLAP cells treated with either TGF-β1 or TGF-β2. CLAP cells were cultured in the presence or absence of 100 pM of either TGF-β isoform and harvested at 12 hour time intervals. Figure 8 shows that significant differences in thymidine incorporation between untreated and TGF-β stimulated cells begin at 36 hours of culture. The affects of the TGF-β isoforms are similar, with TGF-β2 being a slightly more potent stimulator of cell division. At 6 days of culture, differences in thymidine incorporation between treated and untreated cells are highly exaggerated, but there is not a significant difference between the TGF-β isoforms.

Minimal Time Exposure to TGF-β2. Studies were also done to determine the minimal exposure time to TGF-β for the stimulation of increased tritiated thymidine incorporation into DNA of cultured CLAP cells. CLAP cells were cultured in 24-well plates in the presence or absence of TGF-β2. At 2 hour intervals, the cells of one row

of 6 wells were individually collected, rinsed with M199 lacking TGF-β2 and transferred into the wells of a fresh 24-well plate containing TGF-β2-free medium. Cells were then cultured for a total of 48 hours prior to exposure to tritiated thymidine and subsequent DNA extraction. As seen in figure 9, a minimal exposure time of 10-12 hours to TGF-β2 is required by CLAP cells to stimulate thymidine incorporation.

Effects of TGF-β Stimulation on Cell Differentiation. CLAP cells grown in the presence of TGF-β were also examined for their ability to maintain lens-specific characteristics and to express additional differentiation-specific characteristics. CLAP cell were cultured for 5 days in the presence or absence of 1, 10 or 100 pM TGF-β1 or TGF-β2 in serum-free M199. Cells were metabolically labeled with ^{35}S-methionine as described in materials and methods. Autoradiogram of gel containing whole cell lysates is shown in figure 10. TGF-β stimulated an increase in the synthesis of two proteins believed to be spectrin and actin, polypeptides involved with substrate adhesion and a decrease in the synthesis of lens-specific crystallins.

The effects of TGF-β isoforms on the expression of a differentiation-specific marker protein were also examined. Phakinin, a 49 kD cytoskeletal beaded filament protein, accumulates as lens fiber cells mature. CLAP cells were cultured in M199 supplemented with 20% chicken serum in the presence or absence of 100 pM TGF-β1 or TGF-β2. After 7 days of culture, cells were processed for SDS-PAGE and Western blotting. Blots were probed with antisera directed against phakinin. As shown in figure 11, both TGF-β1 and TGF-β2 treated cells lack any detectable phakinin.

DISCUSSION

TGF-β Receptors. The TGF-β receptor is a heteromeric complex composed of

type I and type II receptors and is found in almost every cell type (Wrana, et al., 1994). It was of interest to examine if the receptors for TGF-β were present in the chicken lens. In the present study, we have identified both TGF-β receptors in freshly isolated CLAP cells. TGF-β type I and type II receptors were detected as single bands with approximate molecular weights of 63 kD and 95 kD, respectively. The sizes of both receptors fall within the reported ranges of molecular weights of 53-70 kD for TGF-β receptor type I and 75-110 kD for receptor type II (Massagué, 1992; Derynck, 1994). TGF-β type I and type II receptors were also detected in cultured CLAP cells that were grown as cell aggregates or as monolayers. The presence of both TGF-β receptor types suggests that CLAP cells are capable of detecting and transducing a TGF-β signal intracellularly. The results also imply that our culture conditions do not interfere with TGF-β receptor expression. Although cultured CLAP cells possess the cellular machinery to react to TGF-β stimulation, the elicited responses may not reflect those found *in vivo*.

Neither TGF-β type I nor type II receptors could be detected in freshly isolated superficial fiber cells. It is unlikely that TGF-β receptors do not exist within the membranes of superficial fiber cells. As fiber cells elongate and mature, the amount of plasma membrane increases 1000 fold and the crystallins accumulate to comprise approximately 90% of the total soluble proteins of the lens (Piatigorsky, 1981; Rafferty, 1985). Possibly, due to this tremendous increase in total protein, TGF-β receptors are undetectable in the amount of whole cell lysate which was loaded onto the gels.

Immunohistochemical studies by Obata, et al. (1996) also report positive

staining for TGF-β type I and type II receptors in rat lens epithelial cells and negative staining in the fiber cells. They conclude that the fiber cells are not lacking TGF-β receptors, but that the expression of both receptors is too low to be detected.

Growth and Differentiation. Among the many functions of TGF-β is the control of cell division and terminal differentiation. TGF-β has been shown to inhibit the proliferation of bovine lens epithelial cells (Kurosaka and Nagamoto, 1994). TGF-β can also induce rat lens epithelial explants to extensively elongate, although differently than FGF-induced fiber differentiation (Liu, et al., 1994). Therefore, we hypothesized that TGF-β would function to inhibit the mitosis of cultured CLAP cells and promote the accumulation of differentiated characteristics. The results of this study demonstrated the exact opposite effect. TGF-β stimulation, either in the presence or absence of serum, did not inhibit the proliferation of cultured CLAP cells. Instead, TGF-β stimulated dose-dependent increases in both thymidine incorporation and cellular attachment and spreading. Characteristics of fiber cell differentiation were not enhanced by TGF-β stimulation. Exposure to TGF-β caused a decrease in the synthesis of lens-specific crystallins and no accumulation of phakinin. Thus, the responses of CLAP cells elicited by TGF-β stimulation do not lead to an increase in differentiated characteristics.

Our results contrast those obtained by Kurosaka and Nagamoto (1994) and Nishi, et al. (1996). Kurosaka and Nagamoto report that TGF-β inhibits, in a dose-dependent manner, the growth of cultured bovine lens epithelial cells. Differences in the results obtained by Kurosaka and Nagamoto and those presented here could possibly be explained by differences between species, cell types or culture techniques.

Their experiments were performed using second passaged cells, initially isolated from explants of the central anterior epithelium of bovine lenses. The anterior epithelium of a lens is composed of post-mitotic central epithelial cells, a ring of mitotic stem cells and their post-mitotic daughter cells undergoing the initial stages of terminal differentiation. Explants of the anterior epithelium, therefore, may contain a mixed population of cells. More importantly, the explant contains central epithelial cells which do not normally differentiate into lens fiber cells. By using cultured cells in their second passage, the authors have also inadvertently selected a population of lens cells that have adapted to culture conditions. Nishi, et al. also report the inhibition of proliferation in response to TGF-β2. Their experiments were also performed with primary cultures of human lens anterior epithelial cell explants. In our experiments, we use primary cultures of a pure population of chicken lens annular pad cells that are all post-mitotic and committed to fiber cell formation. Results obtained in our studies demonstrate the responses of a single cell type to the stimulation of TGF-β. TGF-β induced dose-dependent increases in DNA accumulation and thymidine incorporation in CLAP cells cultured in both serum-free and serum-containing conditions. The specific activity of CLAP cells cultured in the absence of serum was higher than that of cells cultured in the presence of serum. This may be explained by the onset of contact inhibition of the growth of cells cultured in the presence of serum (Holley and Kiernan, 1968). Since CLAP cells are a post-mitotic cell population, the TGF-β induced increase in thymidine incorporation and DNA accumulation are most likely unrelated to any potential responses elicited by TGF-β *in vivo*.

Our results agree with those obtained by McAvoy and his group, in that TGF-β

stimulation does not lead to the accumulation of differentiated lens characteristics. In one report, treatment of rat lens explants with TGF-β caused rapid elongation of the lens cells (Liu, et al., 1994). The cells became spindle-shaped, but lacked differentiated traits such as β-crystallin and membrane specializations. Cell death occurred within 3-5 days of culture. In another study, intact rat lenses treated with TGF-β developed subcapsular opaque plaques along the anterior surface of the lenses (Hales, Chamberlain and McAvoy, 1995). The plaques consisted of clumps of abnormal cells, spindle-shaped cells and ECM components. Spindle-shaped cells, layered two to five cells deep, were located between the plaques and were associated with wrinkling of the overlying capsule. Although mitosis was not examined in these studies, the formation of cell clumps and the multilayering of the anterior epithelium provides evidence that TGF-β stimulated abnormal proliferation of lens cells. Again, the differences in the results obtained by McAvoy and those presented here, could possibly be explained by differences between species, cell types or culture techniques. Additionally, in both studies, the lens capsule remained in contact with the entire rat lens epithelium. Therefore, the observed responses might actually result from a mixed population of cell types, or from the combined stimulation of TGF-β and signals originating in the lens capsule. No matter the cause, TGF-β stimulation of rat epithelial cells did not enhance the accumulation of lens fiber characteristics.

Time response to TGF-β. The proliferation of cultured CLAP cells in response to TGF-β stimulation was investigated further by examining the time course of TGF-β1 and TGF-β2 stimulated thymidine incorporation. A significant increase in the specific activity of TGF-β treated cells over control cells was apparent at 36 hours of

continual stimulation. During the times investigated, TGF-β1 and TGF-β2 functioned similarly, with the TGF-β2 isoform always being the more potent stimulator of thymidine incorporation. Since TGF-β2 is the most prevalent isoform found in ocular fluids (Cousins, et al., 1991), the greater response induced by TGF-β2 was not unexpected. At 6 days of culture, both isoforms elicited a significant increase in the specific activity of treated cells that is approximately 10 fold greater than that of control values. The minimum time of exposure to TGF-β2 required to detect an increase in thymidine incorporation in cultured CLAP cells was found to be approximately 10-12 hours. This time frame is similar to times reported in other studies. Although TGF-β1 functions to inhibit the growth of these cell types, the induced response was detected at approximately 10 hours of TGF-β treatment (Howe, Draetta and Loef, 1991; Landesman, et al., 1992; Mülder and Morris, 1992). The similarity in the times required to elicit the inhibition or stimulation of proliferation suggests that the TGF-β receptors detected in cultured CLAP cells are physiologically functional.

Integration of Growth Factor Responses. Chicken lens epithelium contain mRNAs for a variety of different growth factor receptors (Potts, Harocopos and Beebe, 1993), suggesting that lens cells are responsive to multiple growth factor stimulation. In this study, cultured CLAP cells maintained the ability to respond to the various growth factors tested with an increase in the amount of thymidine incorporated into DNA. The ability of these cells to respond to various growth factors suggests that multiple stimulations are involved in the regulation of the development and differentiation of lens epithelial cells.

The ability of one growth factor to regulate cellular responses is dependent upon the presence of other growth factors (Nugent and Edelman, 1992). Studies have revealed cooperativity between various growth factors in eliciting cellular responses. Co-treatment of PDGF and insulin stimulated the growth of neonatal rat lenses more effectively than PDGF stimulation alone (Brewitt and Clark, 1988; 1990). The bFGF-induced fiber cell differentiation of rat epithelial explants has been shown to be enhanced by IGF-1 stimulation (Richardson, Chamberlain and McAvoy, 1993). These studies suggest that normal fiber formation may require specific combinations or sequences of multiple receptor-mediated responses.

Several studies have demonstrated that TGF-β can modify responses elicited by various growth factors. TGF-β inhibits the mitogenic activity of PDGF in cultures of human neonatal fibroblasts by down-regulating the expression of PDGF α-receptors (Paulsson, et al., 1993). TGF-β inhibits the growth of human breast cancer cells by enhancing the expression of insulin-like growth factor-binding proteins (IGFBPs), which regulate the mitogenic effects of IGF (Oh, et al., 1995). TGF-β potentiates the mitogenic activity of bFGF by enhancing interactions between bFGF and FGF receptors (Nugent and Edelman, 1992; Cook, Doumit and Merkel, 1993; Falcone, et al., 1993). Therefore, the *in vivo* cellular responses elicited by TGF-β may not be dependent upon a single receptor-mediated event. Instead, the normal actions of TGF-β *in vivo* probably result from the integration of multiple pathways initiated by several distinct signaling events.

Summary. The results of this study suggest that in culture, TGF-β does not function to keep CLAP cells withdrawn from the cell cycle or promote lens fiber

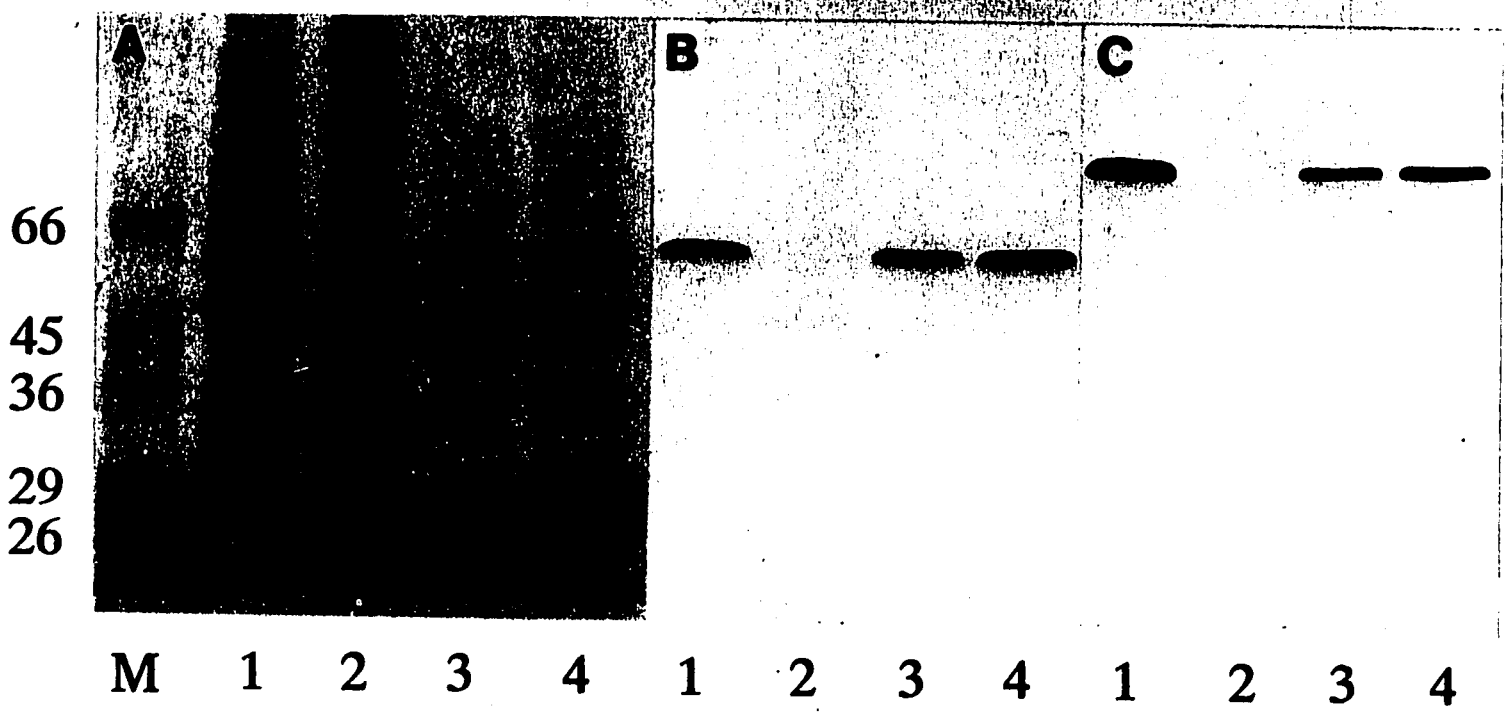
differentiation. On the contrary, TGF-β stimulates cellular spreading and growth which results in the decrease of lens-specific characteristics. The enhanced growth of cultured CLAP cells stimulated by TGF-β may result secondarily from the effects of TGF-β on the production of the ECM. Previous studies in our laboratory have demonstrated that CLAP cells cultured on substrates of ECM molecules show an increase in both thymidine incorporation and cellular spreading (Ireland, Braunsteiner and Mrock, 1993). Several studies report that lens cells placed into culture produce capsular components, such as collagen types I, III and IV; laminin; fibronectin and heparan sulfate proteoglycans (Iwig, et al., 1976; Laurent, et al., 1981; Tassin, Jacquemin and Courtois, 1983; Greenburg and Hay, 1986; Parmigiani and McAvoy, 1991). TGF-β stimulation also induces the production of ECM components in a wide variety of cell types, including lens epithelial cells (Sporn and Roberts, 1992; Attisano, et al., 1994; Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995). Therefore, it may be possible that the enhanced accumulation of ECM stimulated by TGF-β is stimulating the increase in thymidine incorporation of cultured CLAP cells as they attach and spread onto the relevant substratum. Studies have shown that cellular flattening and spreading onto a substrate serves as a strong mitotic stimulus to lens epithelial cells (Glaesser, Rattke and Iwig, 1979; Iwig, Glaesser and Bethge, 1981).

These experiments do not imply that TGF-β functions in the lenticular environment to stimulate cell division. CLAP cells *in vivo* remain in a post-mitotic state and continue to differentiate into lens fiber cells. Cells placed into primary culture are removed from conditions which normally inhibit mitosis and/or promote the acquisition of further differentiated traits. *In vivo*, TGF-β may still inhibit mitosis, but

the elicited responses are integrated into a framework of multiple signals from growth factors, hormones, adjacent cells and the ECM. These studies suggest that normal fiber formation may require specific combinations or sequences of multiple receptor-mediated responses. Therefore, the responses elicited by TGF-β in cultured cells may not reflect the roles TGF-β holds *in vivo*.

Figure 5. Identification of TGF-β type I and type II receptors. Coomassie Blue-stained gel (A) and autoradiograms of corresponding ECL blots probed with antibodies directed against TGF-β receptors type I (B) and type II (C). Each lane contains equivalent amounts of whole cell lysates from freshly isolated CLAP cells (lanes 1), freshly isolated superficial lens fiber cells (lanes 2), CLAP cells cultured as cell aggregates (lanes 3) and CLAP cells cultured as monolayers (lanes 4). Molecular weight standard (M) is in kilodaltons.

IDENTIFICATION OF TGF-β TYPE I AND TYPE II RECEPTORS



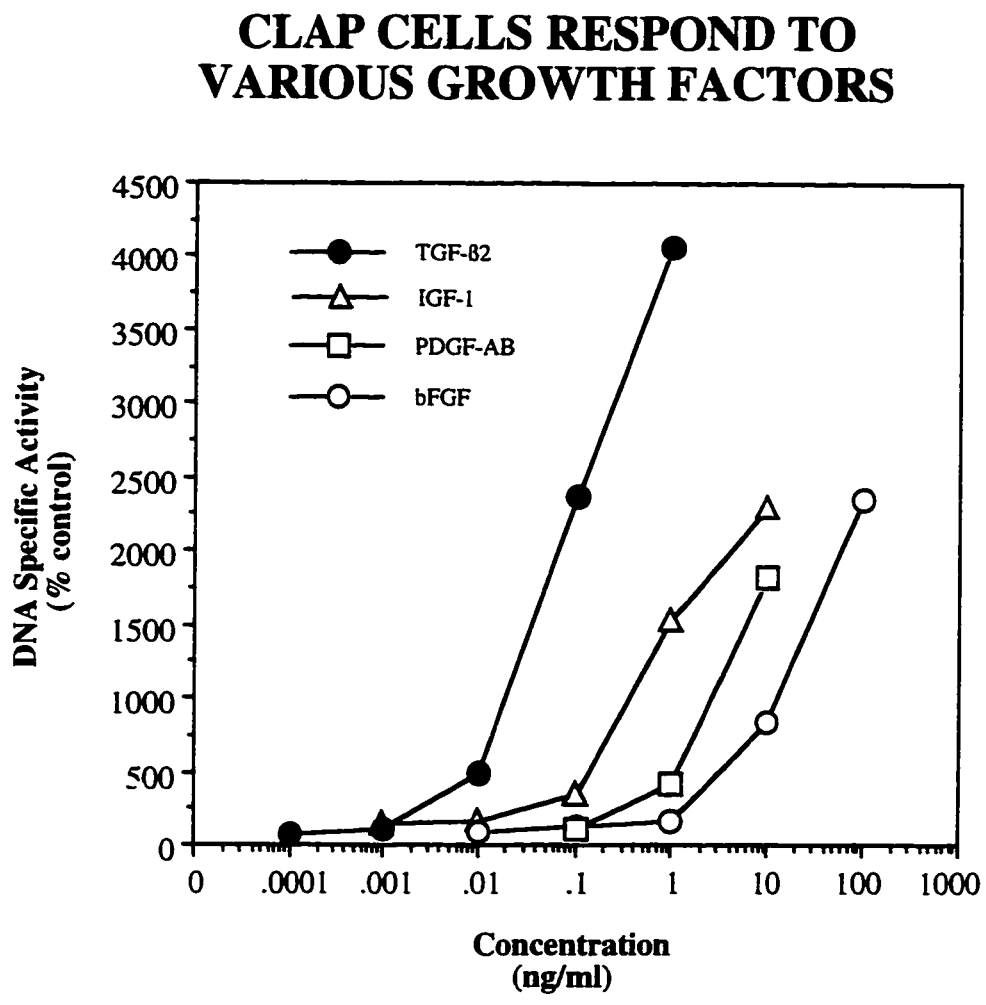
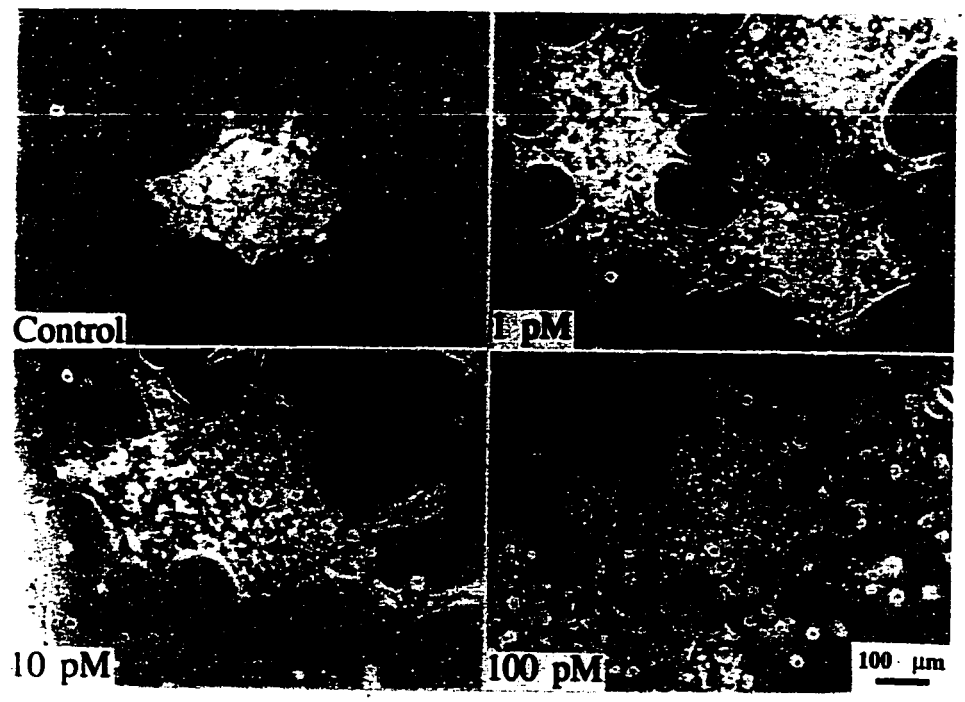


Figure 6 . Effects of various growth factors on the thymidine incorporation of cultured CLAP cells. CLAP cells were cultured for 8 days in the presence of the indicated concentrations of various growth factors. Results are the mean of 12 measurements from 3 different experiments.

Figure 7. Effects of TGF-β stimulation on cellular spreading of cultured CLAP cells. Micrographs of the characteristic spreading of CLAP cells cultured in the absence (A) or presence (B) of 20% chicken serum with the indicated concentrations of TGF-β. Photographs were taken with phase contrast optics on day 2 for cells cultured in the presence of serum and on day 5 for cells cultured in serum-free media.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10 pM
100 pM
100 µm

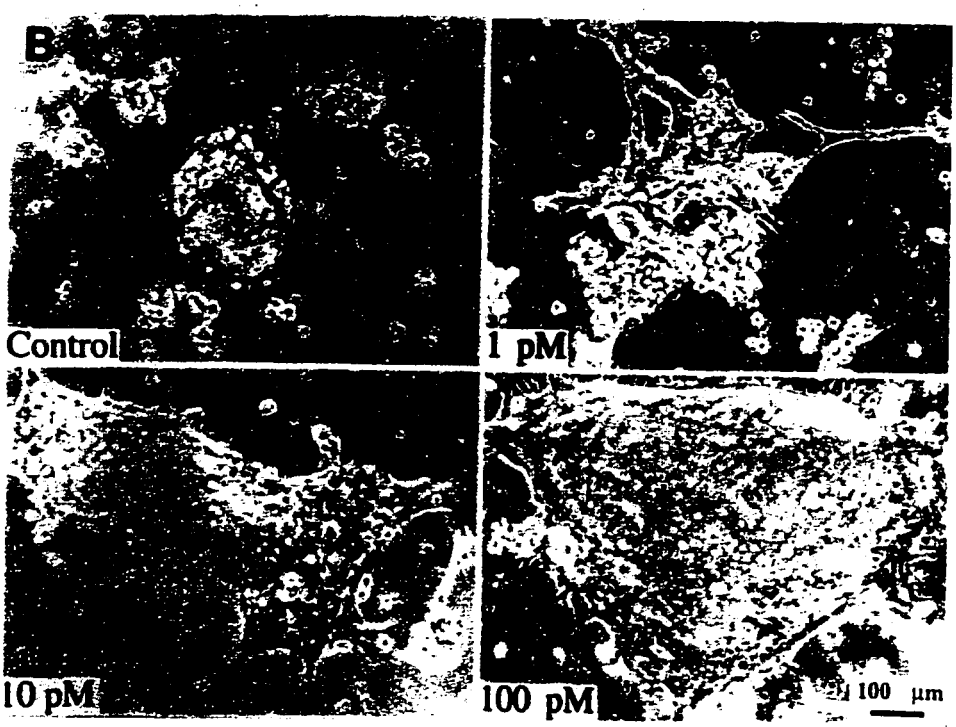

B
Control
1 pM
10 pM
100 pM
100 µm

Table 1. Effects of TGF-β on Cell Growth

A. CLAP cells cultured in 0% serum for 3 days.

Treatment	Protein (μg/well)	DNA (μg/well)	Thymidine Incorporation (cpm/ μg DNA)
None	108±28	0.79±0.12	185±59
TGF-β 1 pM	112±39	0.82±0.13	1399±297
TGF-β 10 pM	129±15	0.86±0.18	2896±743
TGF-β 100 pM	110±14	1.31±0.16	2099±576

B. CLAP cells cultured in 20% serum for 3 days.

Treatment	Protein (μg/well)	DNA (μg/well)	Thymidine Incorporation (cpm/ μg DNA)
None	163±8	0.77±0.06	157±78
TGF-β 1 pM	197±15	0.89±0.05	625±50
TGF-β 10 pM	213±11	1.25±0.31	919±175
TGF-β 100 pM	216±11	1.30±0.12	1242±492

Effects of TGF-β on cell growth. CLAP cells were cultured in serum-free media (A) or in medium containing 20% serum (B) in 24-well plates. Cells were grown in the absence or presence of the indicated concentrations of TGF-β (n=6 for each treatment). Three days after initial plating, three wells were sampled for total cellular protein and three wells were exposed to tritiated thymidine before DNA extraction. Results are the mean ± S.D. and are typical of three additional experiments. Time zero incorporation is 25 ± 7.8.

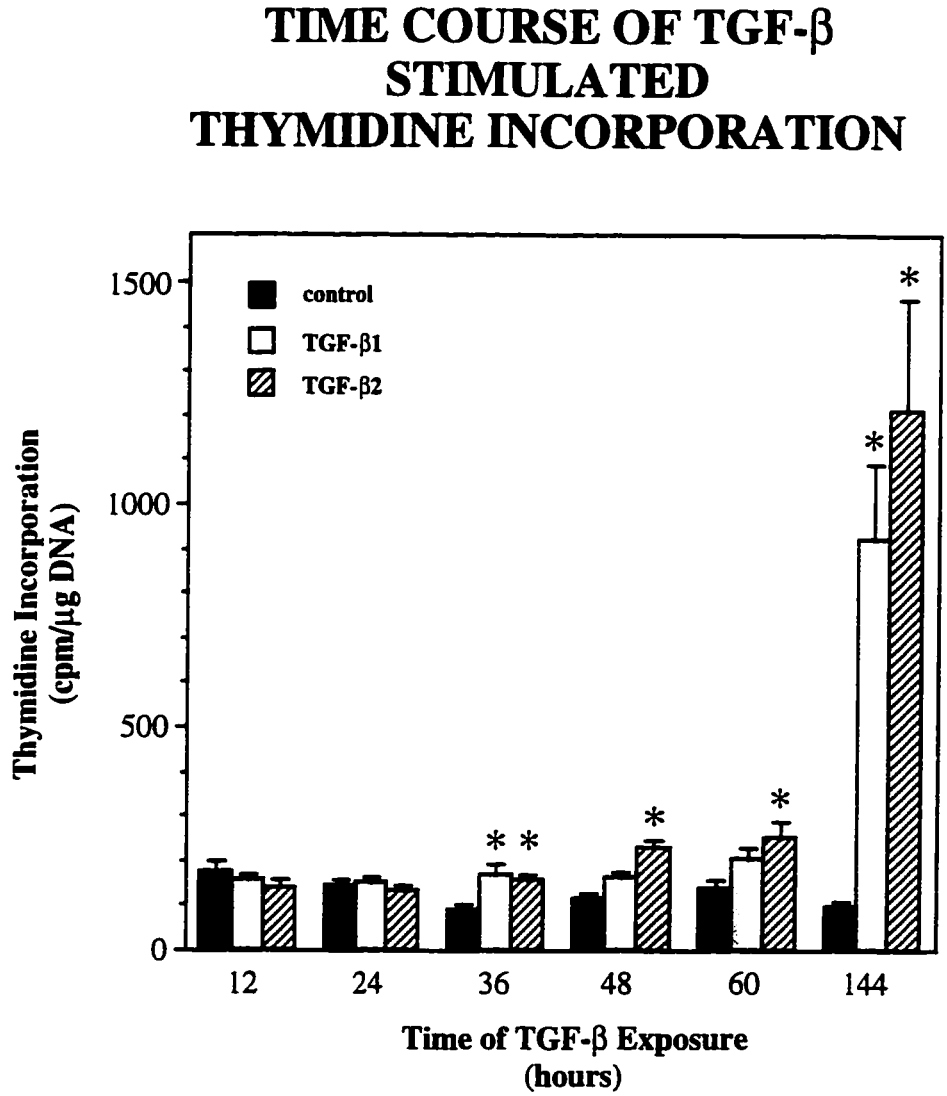


Figure 8. Time course of TGF-β stimulated tritiated thymidine incorporation. CLAP cells were cultured in the absence or presence of 100 pM TGF-β1 or TGF-β2. At the times indicated, cells were exposed to tritiated thymidine. Results are the mean ± S.E. of 12 measurements. * indicates a significant difference from control values at $p < 0.05$.

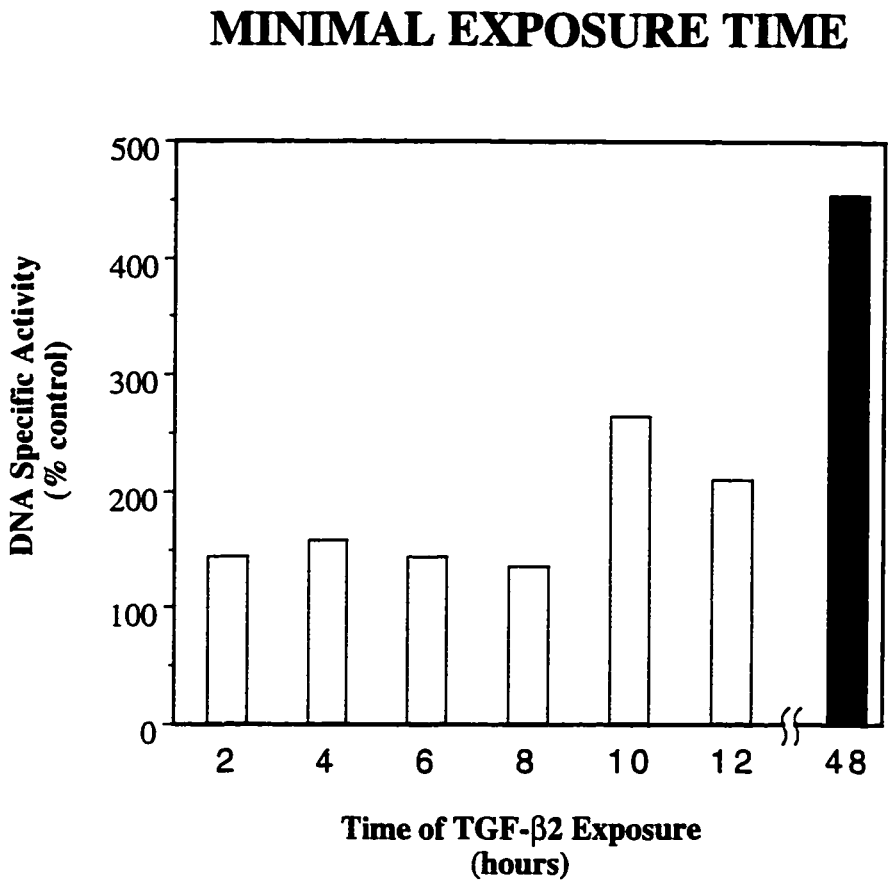


Figure 9. Minimal exposure time for TGF-β2 responsiveness. CLAP cells were cultured in the presence or absence of 100 pM TGF-β2. At the indicated times, cells were collected, rinsed with media lacking TGF-β2 and transferred into a fresh culture dish containing media without TGF-β2. Cells were cultured for a total of 48 hours prior to exposure to tritiated thymidine. Results are the mean of triplicate determinations.

EFFECTS OF TGF-β ON PROTEIN SYNTHESIS

Figure 10. Effects of TGF-β stimulation on CLAP cell protein synthesis. Autoradiogram of 10% gel containing whole cell lysates from metabolically labeled cultured CLAP cells grown with the indicated concentration of TGF-β for 5 days. Equal numbers of radioactive counts were separated. Note the increased specific activity of spectrin and actin polypeptides (upper and lower arrowheads, respectively) in response to TGF-β stimulation. Note also the decrease in specific activity of lens crystallins (*) in response to cellular spreading induced by TGF-β.

EFFECTS OF TGF-β ON DIFFERENTIATION

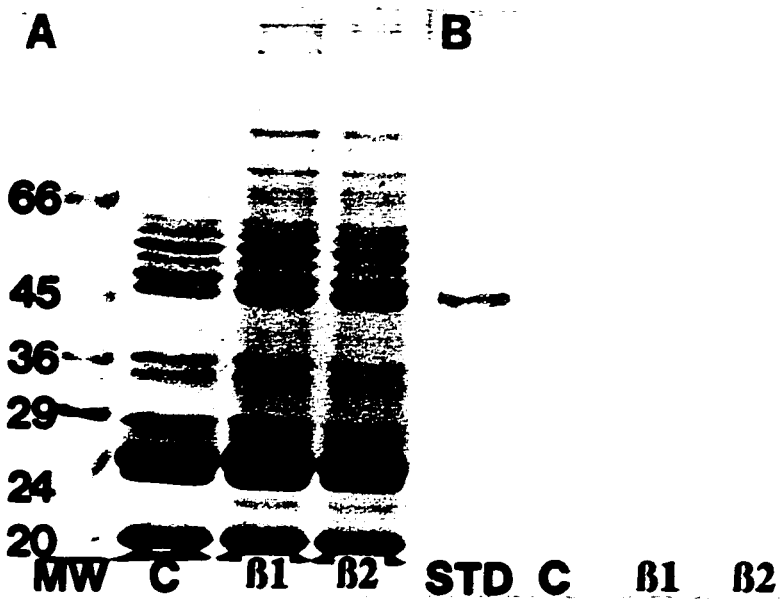


Figure 11. Effects of TGF-β stimulation on CLAP cell differentiation. SDS-PAGE (A) and Western blot (B) of whole cell lysates of CLAP cells cultured in 100 pM of the indicated TGF-β isoforms (C=control). Blot was probed with antibodies directed against a 49 kD lens fiber cytoskeletal protein. Cells were grown for 7 days in 20% chicken serum. Molecular weight standard (MW) is in kilodaltons. (STD) is the 49 kD protein present in chick lens fiber cells. Note absence of any detectable 49 kD protein in TGF-β treated cells.

Chapter 3.

The Effects of TGF-β on Extracellular Matrix (ECM) Production by Cultured CLAP Cells and the Effects of ECM Components on the Responses Elicited by TGF-β.

INTRODUCTION

The terminal differentiation of lens fiber cells may be under the control of genetic mechanisms or morphogenic cues located within the microenvironments which encompass the lens cells. As the differentiating lens fibers migrate along the lens capsule, they encounter regional differences in the composition and architecture of their surrounding matrix (Kuwabara, 1975; Johnson and Beebe, 1984; Mohan and Spiro, 1986; Webster Jr., et al., 1987). The differences found within the lens capsule could result from the preferential production/modification of the capsule as it is being deposited by the lens cells themselves (Young and Ocumpaugh, 1966; Rafferty and Goossen, 1978; Fitch, Mayne and Linsenmayer, 1983; Laurent, et al., 1987). During their migration, lens cells also encounter different ocular fluids. The anterior of the lens contacts the aqueous humor, the equatorial region apposes the ciliary body and the posterior surface is bathed in vitreous humor. The aqueous and vitreous humors vary in their ability to influence lens cell behavior (Coulombre and Coulombre, 1963; Lovicu, Chamberlain and McAvoy, 1995). Thus, the heterogeneity existing between the different regions of the lens capsule could be the result of soluble factors found within the aqueous or vitreous humors which signal the lens cells to alter their extracellular matrix (ECM). Alternatively, the matrix may differ due to soluble matrix-degrading enzymes found preferentially within the different regions of lens capsule. Regardless of the causes, the variations found between the regions of the lens capsule could function to direct or sustain lens cell migration, provide morphogenetic cues which participate in fiber cell differentiation or serve as sites for the binding and presenting of factors responsible for stimulating lens cell differentiation. Thus, the

differentiation of lens cells could potentially be regulated by the composition of the ECM and the presence of soluble factors located within the ocular fluids. The soluble factors could originate from the various surrounding ocular tissues or from the lens cells themselves. Lens cells have been shown to exhibit endogenous levels of growth factors (Schweiger, et al., 1988; Caldes, et al., 1991; Pelton, et al., 1991; Tripathi, et al., 1991; Majima, 1995; Nishi, et al., 1996).

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine found in a large variety of cells and tissues. Lens epithelial and fiber cells plus both aqueous and vitreous humors contain TGF-β (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Potts, Bassnett and Beebe, 1995). TGF-β has the ability to bind heparan sulfate proteoglycans, fibronectin and type IV collagen, suggesting that it could also be a component of the lens capsule (Fava and McLure, 1987; Boyd, et al., 1990; Paralkar, Vukicevic and Reddi, 1991; Attisano, et al., 1994). TGF-β functions not only to regulate cell proliferation and differentiation, but also to influence the secretion of ECM proteins and their degrading proteinases (Sporn and Roberts, 1992; Attisano, et al., 1994). TGF-β stimulation may also regulate the selective expression of specific integrin subunits (Ignotz and Massagué, 1987; Heino, et al., 1989). The specific combination of the various integrin subunits determines the specificity of the interactions between the cell and a particular component of the ECM. Therefore, TGF-β has the ability to affect cell functioning both directly and indirectly. TGF-β indirectly regulates cellular behavior through the modification of the ECM and by the alteration of a cell's ability to interact with the ECM constituents. TGF-β directly regulated cell functioning through signals transduced into a cell's interior through a

heteromeric complex of two cell surface receptors. Both TGF-β type I and type II receptors need to be functional in order for TGF-β to produce an intracellular signal (Derynk, 1994).

The model we have developed to examine the influences of TGF-β on lens cells involves the primary culture of post-mitotic epithelial cells committed to fiber formation. These cells are obtained from an epithelial specialization found in chicken lenses known as the annular pad (Hanna and Keats, 1966). Studies have demonstrated that TGF-β stimulation of lens cells results in the accumulation of ECM molecules (Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995). Previous experiments performed in our laboratory have shown that the presentation of an ECM substratum stimulates an increase in the proliferation of cultured CLAP cells (Ireland, Braunsteiner and Mrock, 1993). Therefore, these studies were done to examine 1) if TGF-β stimulation can affect the production of ECM molecules by cultured CLAP cells and 2) if the presence of an ECM substratum could affect TGF-β induced responses of cultured CLAP cells.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, all reagents were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Tissue culture media was purchased from Celox (Hopkins, MN, U.S.A.); tissue culture supplements from GIBCO BRL (Grand Island, NY, U.S.A.); plasticware from Corning Glass Works (Corning, NY, U.S.A.). TGF-β1, murine laminin, murine collagen type IV, human fibronectin plus the polyclonal antibodies directed against these ECM components were purchased from Becton/Collaborative Research, Inc. (Bedford, MA, U.S.A.). TGF-β2 was purchased

from Genzyme Corporation (Cambridge, MA, U.S.A.). Nitrocellulose membrane and BCIP/NBT color developing reagents were obtained from BIO-RAD Laboratories (Hercules, CA, U.S.A.); WXR-B X-ray film from Wolverine X-Ray Sales and Service Inc. (Detroit, MI, U.S.A.). Tritiated thymidine was purchased from ICN Radiochemicals (Costa Mesa, CA, U.S.A.).

Cell Isolation and Culture. Chicken lens annular pad (CLAP) cells were isolated from 2- to 3-month-old chickens as previously described (Ireland, Tran and Mrock, 1993) with minor modifications. Three hundred micrograms of total protein were loaded into each well of 24-well tissue culture dishes. Wells contained 1 ml Medium-199 (M199) supplemented with penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and 0.1% fungizone, but lacked serum. Cells were viewed and photographed with phase contrast optics.

Extracellular Matrix Coatings. Prior to the addition of cells, tissue culture plates were coated with ECM components as previously described (Ireland, Braunsteiner and Mrock, 1993). Laminin, fibronectin or type IV collagen were suspended in sterile PBS, added to the wells of 24-well culture dishes and allowed to incubated overnight at 37°C, yielding a final coating concentration of 1 or 10 $\mu g/cm^2$. The solutions were aspirated and the wells washed several times in culture media before the addition of cells.

Culture treatments. Cells were treated with TGF-β1 or TGF-β2 immediately after being plated and every other day thereafter. Concentrated stocks of the growth factors were added directly to the culture medium to yield the indicated dosages. Each dosage was examined 6 times per experiment. Each experiment was repeated at least

three times. Controls received no treatments.

Thymidine incorporation, DNA and protein determination. At indicated times, the conditioned media was retrieved for gel electrophoresis and the cells were processed for tritiated thymidine incorporation and DNA accumulation. Procedures for both tritiated thymidine incorporation and DNA determination have been previously published by Ireland, Tran and Mrock (1993). For protein determination, aliquots of concentrated conditioned culture media were acid precipitated, solubilized in NaOH and quantified against a BSA standard as previously described (Ireland and Jacks, 1989).

Gel electrophoresis and Western blotting of secreted matrix proteins. Conditioned media was concentrated approximately 20-fold by centrifugation in microconcentrators purchased from Amicon Inc. (Beverly, MA, U.S.A.). Triplicate aliquots of the concentrated media were taken for total protein determination. The remaining concentrated media was mixed with equal volumes of 2X Laemmli sample buffer and subjected to sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) on 5% gels (Laemmli, 1970). Gels were either silver stained or stained with 0.25% Coomassie Brilliant Blue R solution to visualize separated proteins. Samples on duplicate gels were electrophoretically transferred to nitrocellulose sheets for Western blotting.

Western blots were probed with commercially available polyclonal antibodies directed against murine type IV collagen, human fibronectin or murine laminin. Secondary antibodies were conjugated to alkaline phosphatase and color developed with BCIP/NBT reagents according to protocols provided by manufacturer.

ECM deposition. At indicated times, culture media was aspirated from the wells. The remaining CLAP cell layer was dissolved by a 15 minute exposure to 0.5% Triton X-100 in PBS, followed by a three minute exposure to 0.025 N NH_4OH and three rinses with PBS (Fridman, et al., 1985). ECM components deposited by CLAP cells were collected by the addition of sample buffer directly into the well. Wells were scraped with a rubber policeman and sample buffer containing the ECM molecules was transferred into microcentrifuge tubes. Samples were separated by SDS-PAGE on 5% gels and gels were then silver-stained and dried for permanent records.

Statistical analysis. Two-factor analysis of variance (ANOVA) was used for statistical analysis of data. A level of $p < 0.05$ was accepted as statistically significant.

RESULTS

Detection of secreted ECM molecules by Western blotting. TGF-β influences the ECM production of many cell types, including rat lens epithelial cells (Sporn and Roberts, 1992; Attisano, et al., 1994; Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995). These studies were conducted to examine if TGF-β1 or TGF-β2 could stimulate CLAP cells to synthesize and secrete ECM molecules. Thus, CLAP cells were cultured for 5 days in M199 containing 100, 10 or 1 pM TGF-β1 or TGF-β2. Concentrated conditioned media was subjected to SDS-PAGE and Western blotting. Blots were probed with polyclonal antibodies directed against murine collagen type IV, murine laminin or human fibronectin. As seen in figure 12, both TGF-β1 and TGF-β2 induced dose-dependent increases in the accumulation of collagen type IV and fibronectin into the culture media. TGF-β2 seemed to be more

effective than TGF-β1 at stimulating the secretion of these ECM components. Although, positive staining of a murine laminin standard was observed, the secretion of any laminin into the culture media was never detected, by the various antibodies used. (Data not shown).

Detection of deposited ECM molecules. We were also interested in examining the ECM components that were deposited onto the tissue culture plastic by cultured CLAP cells. CLAP cells were cultured in the presence or absence of 100 pM TGF-β1 for 5 days. The deposited ECM components were collected in sample buffer after the culture media was removed and the remaining cell layer was dissolved. Figure 13 is a silver-stained 5% gel of the deposited ECM from such an experiment. Four bands need to be mentioned. Proteins produced by both control and TGF-β treated CLAP cells are marked with small arrows. An unidentified protein band with approximate molecular weight of 400 kD (upper small arrow) appears in similar amounts in both treated and untreated lanes. TGF-β stimulated a slight increase in the production of a protein with the approximate molecular weight of 175 kD, similar to the molecular weight of collagen type IV (lower small arrow). But, more interestingly, TGF-β also stimulated the production of two proteins that are not found within the ECM deposited by untreated CLAP cells (large arrows). The first is an unidentified protein with extremely high molecular weight (top large arrow), which may represent TGF-β induced production of heparan sulfate proteoglycans (HSPG). The second protein, induced by TGF-β stimulation, has the molecular weight of approximately 220 kD (bottom large arrow). Based on the observations that the 220 kD band co-migrates with, and silver-stains the same color as the fibronectin standard, it is believed to be

fibronectin.

Effects of ECM on TGF-β1 induced responses. CLAP cells *in vivo* remain in continuous contact with the lens capsule as they migrate posteriorly. Thus, the lens capsule could be influencing the behavior of the lens cells by modulating the effects of specific signaling factors like TGF-β. We were, therefore, interested in examining the possible effects of specific ECM molecules on TGF-β induced responses in cultured CLAP cells. We coated 24-well culture dishes with specific ECM molecules as described in materials and methods. CLAP cells were plated onto either the tissue-culture plastic or onto the coated plastic and cultured in the absence or presence of 10 pM TGF-β1. Responses assayed include the amount of tritiated thymidine incorporation and the extent of cellular spreading and attachment, as viewed by phase contrast microscopy.

SPREADING. The typical differences in spreading and attachment of CLAP cells cultured under the various conditions are shown in figure 14. CLAP cells grown on plastic in the presence of TGF-β1 (figure 14A) display enhanced cellular attachment and spreading as compared to unstimulated cells cultured on plastic (figure 14E), which remain as aggregated cell masses with occasional signs of attachment. CLAP cells plated onto collagen type IV coated wells and stimulated with TGF-β1 exhibit monolayer growth (figure 14B). CLAP cells grown on collagen type IV in the absence of TGF-β showed a degree of spreading very similar to TGF-β1 treated cells cultured on plastic (figure 14F). CLAP cells grown on a substrate of fibronectin and receiving TGF-β1 stimulation exhibit monolayer growth (figure 14C). Approximately half the cells cultured on a fibronectin substratum without TGF-β stimulation (figure

14G) remain as cell aggregates, but are firmly attached to the fibronectin substratum. The remaining half of the cells attach and spread to a greater degree than untreated cells grown on plastic, but not to the extent of TGF-β1 treated cells grown on plastic. Laminin, coated at 10 $\mu g/cm^2$, stimulated enhanced spreading and attachment as compared to untreated CLAP cells cultures on plastic (figure 14H). Cells stimulated with TGF-β1 and plated on laminin also grew into monolayers (figure 14D). These observations suggest that both TGF-β and individual ECM molecules can independently induce cultured CLAP cells to adhere and spread to a greater extent than cells cultured without either treatment. An interaction between TGF-β and ECM stimulation is also evident. CLAP cells receiving simultaneous stimulation from both TGF-β and ECM display growth far beyond cells receiving either individual stimuli.

THYMIDINE INCORPORATION. Thymidine incorporation into DNA was examined to determine the mitotic activity of CLAP cells cultured under the conditions described above. Figure 15 shows the typical levels of thymidine incorporation of CLAP cells that have been cultured on tissue-culture plastic or on the indicated ECM molecule either in the presence or absence of 10 pM TGF-β1. Collagen type IV: Exposure to either 10 pM TGF-β or collagen type IV caused an increase in tritiated thymidine incorporation into DNA of approximately 170% over control values. TGF-β1 stimulation of CLAP cells grown on a collagen type IV substrate resulted in an additive increase in thymidine incorporation (309% stimulation over control levels). Fibronectin: CLAP cells cultured on fibronectin or on plastic in the presence of 10 pM TGF-β1 displayed an increase in thymidine incorporation over untreated cells cultured on plastic of 306% or 753%, respectively. CLAP cells cultured on a

fibronectin substratum and stimulated with TGF-β had a synergistic increase in thymidine incorporation of 2341%. Laminin: Cells plated on laminin displayed an increase in tritiated thymidine incorporation of 258% over untreated cells cultured on plastic. TGF-β1 stimulation of CLAP cells cultured on plastic caused an increased in thymidine incorporation of 1378% of control. CLAP cells cultured on a substrate of laminin and stimulated by TGF-β1 had a synergistic increase in thymidine incorporation of 3180%. The reported levels of thymidine incorporation for CLAP cells cultured on plastic in the presence of TGF-β differed between the specific sets of experiments. The disparity is the result of 1) variations in the viability of the different isolations of cells which were placed into primary culture and 2) variations in the lengths of time that the cells were cultured. Results therefore could not be pooled and compared. Comparisons were made between controls and experimental data from the same set of experiments.

This data confirms that ECM or TGF-β alone can influence cellular responses of cultured CLAP cells. It also demonstrates that the ECM and TGF-β interact in a synergistic manner to greatly enhance both thymidine incorporation and cellular spreading. Data from experiments in which CLAP cells were grown on collagen type IV in the presence of TGF-β revealed an additive increase in thymidine incorporation. This is due to the termination of the experiments on day 4 of culture. If these experiments were terminated on day 6, as with the experiments with laminin and fibronectin, it is believed that a synergistic increase would be observed.

DISCUSSION

Effects of TGF-β on ECM Production. Lens epithelial and fiber cells, *in vivo*,

secrete the ECM molecules that form the lens capsule (Young and Ocumpaugh, 1966; Rafferty and Goossen, 1978; Fitch, Mayne and Linsenmayer, 1983; Laurent, et al., 1987). The major component of the capsule is collagen type IV (Cammarata and Spiro, 1985). The non-collagenous constituents of the capsule include heparan sulfate proteoglycans and the glycoproteins entactin, laminin and fibronectin. Although the lens capsule is a continuous basement membrane, it's composition and architecture differs between the anterior and posterior surfaces (Kuwabara, 1975; Johnson and Beebe, 1984; Mohan and Spiro, 1986; Webster Jr., et al., 1987). These differences could be the result of preferential production of specific ECM components by the anterior lens epithelium, the differentiating equatorial cells and the posterior lens fiber cells. The distinct lens cell types may receive identical stimuli to synthesize ECM; yet, depending upon their state of differentiation, they may actually produce different ECM constituents (Vollberg Sr., George and Jetten, 1991). Alternatively, the individual cell types could produce the assorted ECM molecules in response to dissimilar signals located within the distinct microenvironments. These variations in the structure of the lens capsule may play important roles in directing the terminal differentiation of lens cells.

Several studies have demonstrated that cultured lens epithelial cells also produce ECM molecules. Cultured bovine and murine lens epithelial cells synthesize collagen type IV (Laurent, et al., 1981; Muggleton-Harris and Higbee, 1987). Explants of chicken lens anterior epithelium and cultured human lens epithelial cells have been shown to secrete both collagen type IV and laminin (Greenburg and Hay, 1986; Arita, et al., 1993), while anterior epithelial explants from neonatal rat lenses

produce only laminin (Parmigiani and McAvoy, 1991). Cultured bovine lens epithelial cells also synthesize fibronectin and collagens type I and type III (Laurent, et al., 1981; Tassin, Jacquemin and Courtois, 1983), while explants of human lens anterior epithelium produced collagens type I, IV, V and VI (Nishi, et al., 1995).

The production of ECM by cultured lens cells could reflect a normal function of lens cells or may represent a general wounding phenomena of cultured cells. Cells placed in an inadequate environment such as on tissue culture plastic attempt to produce a new matrix and enhance cell-matrix interactions, which in turn serves to maintain cell survival in culture (Delcommenne and Streuli, 1995). Alternatively, the production of ECM components by cultured lens cells may be in response to serum stimulation, since serum contains a mixture of several growth factors known to affect the behavior of lens cells. The production of specific ECM molecules in response to stimulation by a particular growth factor has not been studied extensively. FGF has been shown to stimulate rat lens epithelial explants to produce a capsule-like ECM which immunologically stained for laminin and HSPG (Lovicu, Chamberlain and McAvoy, 1995). EDGF, which has been identified as FGF, stimulates the production of collagen type IV by cultured bovine lens epithelial cells (Kern, et al., 1983; Tassin, Jacquemin and Courtois, 1983). Nishi, et al. (1996) reports that bFGF as well as TGF-β can stimulate cultured human lens epithelial cells to secrete collagen. TGF-β stimulation of rat lens epithelial explants induced the production of collagen type I, laminin and HSPG (Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995).

Since TGF-β has been found within the ocular environment, it is potentially available to lens cells and could function *in vivo* to stimulate the production of the

various ECM components of the lens capsule (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995; Potts, Bassnett and Beebe, 1995). In this study, we examined the possible effects of TGF-β on the secretion of lens capsule components. With the use of polyclonal antibodies directed against specific ECM proteins, we have shown that TGF-β stimulation of cultured CLAP cells induces the secretion of collagen type IV and fibronectin. Collagen type IV and fibronectin were not detected in the culture media collected from untreated cells. Additionally, the production of laminin was never observed, even with the use of an assortment of antibodies directed against this abundant capsular glycoprotein. Our inability to detect the secretion of laminin may be explained by a lack of cross-species reactivity of the antibodies.

We also examined, with the use of silver-stained SDS-PAGE gels, the matrix deposited onto the bottom of the culture dish. The ECM deposited by cultured CLAP cells in response to TGF-β differs from the matrix produced by untreated cells. TGF-β induced the production of an unidentified protein with extremely high molecular weight. This protein may represent the TGF-β induced production of HSPG. TGF-β has been reported to stimulate the production of HSPGs by rat lens epithelium (Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995). The posterior lens capsule contains a greater concentration, and a larger species, of HSPG than the anterior lens capsule (Mohan and Spiro, 1986). It has been shown that TGF-β stimulates the production of HSPGs with higher molecular weights than the HSPGs produced by untreated fibroblasts (Nugent and Edelman, 1992). Thus, the preferential production of larger HSPGs in the posterior capsule could result from TGF-β stimulation of

terminally differentiating lens epithelial cells.

TGF-β also stimulated the production of a 220 kD protein believed to be fibronectin. The observation that TGF-β stimulates the production of fibronectin by cultured CLAP cells was of interest, since fibronectin is the least abundant protein found within the bovine lens capsule (Cammarata and Spiro, 1985) and has been reported to be completely absent from adult rat capsules (Parmigiani and McAvoy, 1991). The TGF-β induced synthesis of fibronectin could function to influence the motility of the differentiating lens cells. Fibronectin has been shown to enhance the migratory potential of keratinocytes and epithelial cells (O'Keefe, et al., 1985; Nickoloff, et al., 1988). Additional migration studies of cultured rabbit lens epithelial cells also demonstrate that fibronectin promoted the maximal migration in assays where cells migrated onto surfaces coated with the protein and in assays where fibronectin was added to the culture media (Olivero and Furcht, 1993). These studies are in contrast with the results obtained by Parmigiani and McAvoy (1991). They report that epithelial cells obtained from explants of embryonic and neonatal rat lenses lose their ability to migrate onto a fibronectin substrate as the animal ages; while cells of all ages retain the ability to migrate onto laminin. The conflicting results on the role of fibronectin in cell migration implies that multiple ECM molecules are involved with the *in vivo* migration of lens cells. Although fibronectin comprises only a small portion of the capsular constituents, the concentration of the protein is highest in the posterior capsule (Cammarata and Spiro, 1985; Mohan and Spiro, 1986). It is possible that the TGF-β induced production of fibronectin may slightly alter the composition of the posterior lens capsule and thereby enhance the migration of the lens fiber cells.

The data presented here demonstrates that TGF-β is capable of stimulating the production of ECM molecules, subtly changing the lens capsule and potentially modifying the behavior of lens cells.

Effects of TGF-β and the ECM on growth. TGF-β could function *in vivo* to modify the lens capsule which may facilitate migration or sustain terminal differentiation. In turn, the lens capsule could be influencing the behavior of the lens cells by modulating their responses to specific signaling factors like TGF-β. Therefore, we wanted to examine the possible effects of specific ECM molecules on TGF-β induced responses of cultured CLAP cells. The results reported here demonstrate that stimulation by TGF-β or individual ECM proteins can independently increase the attachment/spreading and thymidine incorporation of cultured CLAP cells. Simultaneous stimulation from both TGF-β and an ECM substratum interacted in a synergistic manner to greatly enhance the same responses. Therefore, the responses of CLAP cells to TGF-β can be modulated by the extracellular environment.

Simultaneous stimulation of CLAP cells by both TGF-β and an ECM substratum resulted in a synergistic response, implying an unknown interaction between the two signaling pathways. The interaction may be as simple as the proper presentation of the TGF-β molecule to CLAP cells by an ECM component or much more complex. A possible common link to both signaling pathways are the integrins. Integrins are cell surface adhesion receptors for ECM components, which couple the extracellular environment to the cytoskeleton of the cell (Hynes, 1992). Therefore, integrins not only function to attach a cell to its matrix, but also to transduce extracellular signals that stimulate similar responses to those elicited by growth factors.

Data also suggests that integrins can activate signaling cascades which interact with growth factor signaling pathways, thereby modulating cellular responses (Schlaepfer, et al., 1994; Sastry, et al., 1996).

Individual integrins are heterodimers of covalently linked α and β subunits. TGF-β can specifically and selectively regulate the expression of integrin subunits (Heino and Massagué, 1989; Heino, et al., 1989). The specific combinations of the various subunits determines the specificity of interactions with particular extracellular matrix components and possibly the specific cellular response. TGF-β also regulates ECM production/modification. Recently, Delcommenne and Streuli (1995) demonstrated that the nature of the ECM could control the expression of integrin subunits. Any change in the ECM might alter the expression of integrin subunits which, in turn, could modify cell behavior.

In our culture system, TGF-β stimulation or the presence of an ECM substratum induced the proliferation of cultured CLAP cells. Although not examined in this report, the presence of an ECM substratum or TGF-β stimulation could also modulate CLAP cell expression of integrins. Integrins could then modify cell behavior by enhancing cell division. Therefore, the synergistic increase in thymidine incorporation and the monolayer growth observed in CLAP cells cultured on an ECM substrate in the presence of TGF-β may result from a combination of 1) direct stimulation from TGF-β, 2) direct signaling from the ECM substratum and 3) additional stimulation elicited by alterations of integrin subunit expression induced by the presence of TGF-β and the ECM.

Summary

A major function of TGF-β is the regulation of ECM production. TGF-β in the ocular environment may function to stimulate lens cells to produce the lens capsule. Our data shows that TGF-β stimulation of cultured CLAP cells does induce the production of ECM components and may contribute to the production/modification of the lens capsule.

Data presented here also demonstrates increased thymidine incorporation and enhanced cellular spreading of CLAP cells cultured on an ECM substrate or in the presence of TGF-β. Synergistic increases in both responses were induced by the simultaneous stimulation of TGF-β and an ECM substrate. These elicited responses of cultured CLAP cells must be unrelated to the responses occurring within the lenticular environment. CLAP cells, *in vivo*, remain in contact with the lens capsule and still continue to be post-mitotic. The ECM components within the lens capsule are not signaling the lens cells to divide. Likewise, a normal function of TGF-β in the lens cannot be the stimulation of mitosis. We believe that TGF-β, *in vivo*, may still inhibit mitosis, but also function to modify the lens capsule. The alteration of the capsule may serve to promote cell migration or to alter the activity of matrix-bound growth factors. In this study, we confirm that TGF-β can stimulate cultured CLAP cells to produce ECM proteins and that the ECM is capable of influencing TGF-β mediated responses.

EFFECTS OF TGF-β ON ECM SECRETION

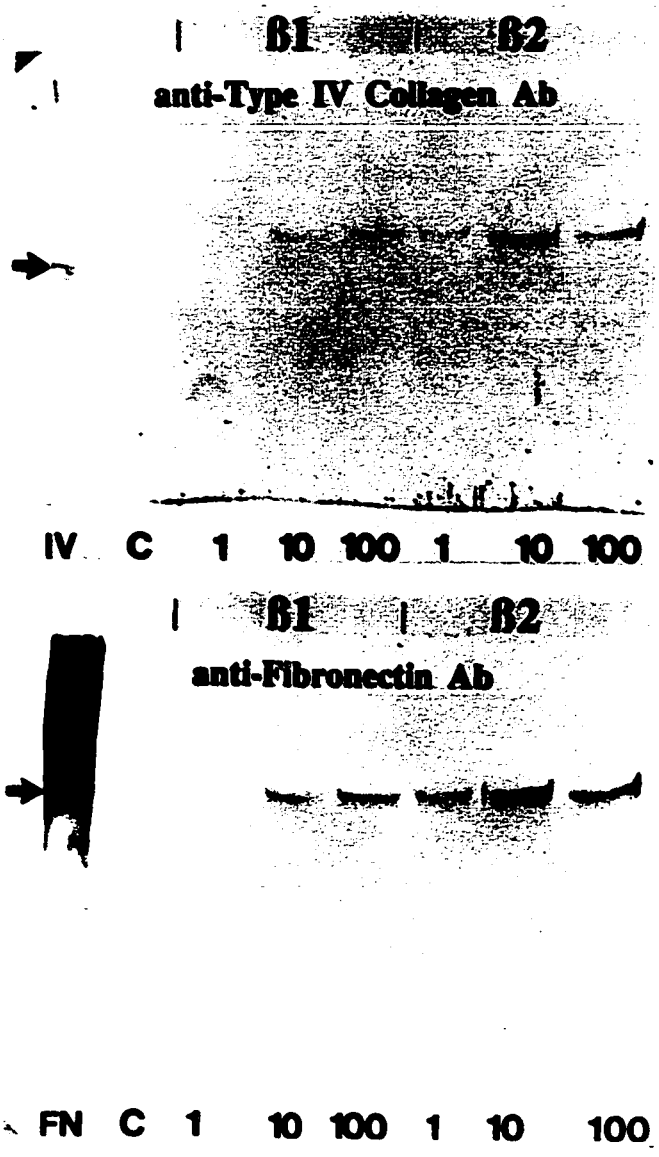


Figure 12. Dose-dependent effects of TGF-β isoforms on the secretion of ECM components. Western blots of 5% SDS-PAGE gels probed with polyclonal antibodies directed against murine collagen type IV (1:1000) or human fibronectin (1:2000). Lanes contain equivalent amounts of protein from culture media of CLAP cells grown for 5 days in the presence of the indicated concentrations of either TGF-β isoform. (C) contains culture media from untreated cells. Arrows indicated the positions of the standards for collagen type IV (IV) and fibronectin (Fn).

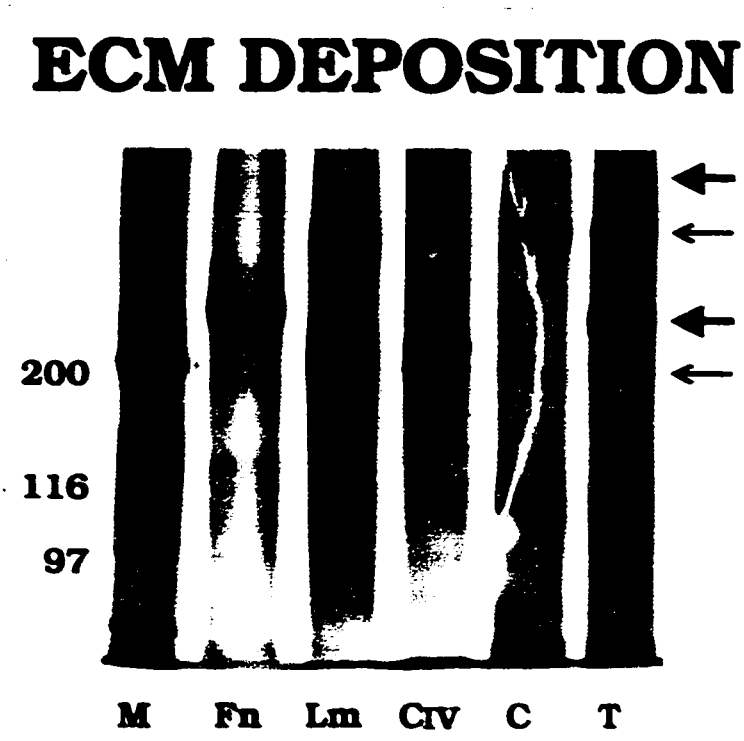


Figure 13. Effects of TGF-β1 on the deposition of ECM molecules. Silver-stained 5% gel CLAP cells cultured in the presence (T) or absence (C) of 100 pM TGF-β1 for 5 days. Standards for ECM molecules are as follows: (Fn) fibronectin; (Lm) laminin; and (CIV) collagen type IV. Molecular weight standard (M) is in kilodaltons. Small arrows mark proteins produced by both control and TGF-β treated CLAP cells. Large arrows indicate proteins deposited into the ECM in response to TGF-β stimulation.

Figure 14. Effects of ECM substrates of TGF-β1 stimulated CLAP: enhanced attachment and spreading. Micrographs of CLAP cells plated directly onto the tissue-culture plastic (A and E) or onto plastic coated with collagen type IV (B and F), fibronectin (C and G), or laminin (D and H). The final coating concentration of collagen type IV and fibronectin was 1 $\mu g/cm^2$. Laminin was coated at 10 $\mu g/cm^2$. Cells were cultured in the presence (A-D) or absence (E-H) of 10 pM TGF-β1 for a total of 4 days on the collagen substrate and 6 days on a substrate of fibronectin or laminin. Control cells (A and E) showed no change in attachment between days 4 and 6. Untreated cells cultured on plastic (E) remain as loosely attached cell aggregates. Cells grown on fibronectin in the absence of TGF-β (G) remain as cell aggregates but are firmly attached to the underlying substrate.

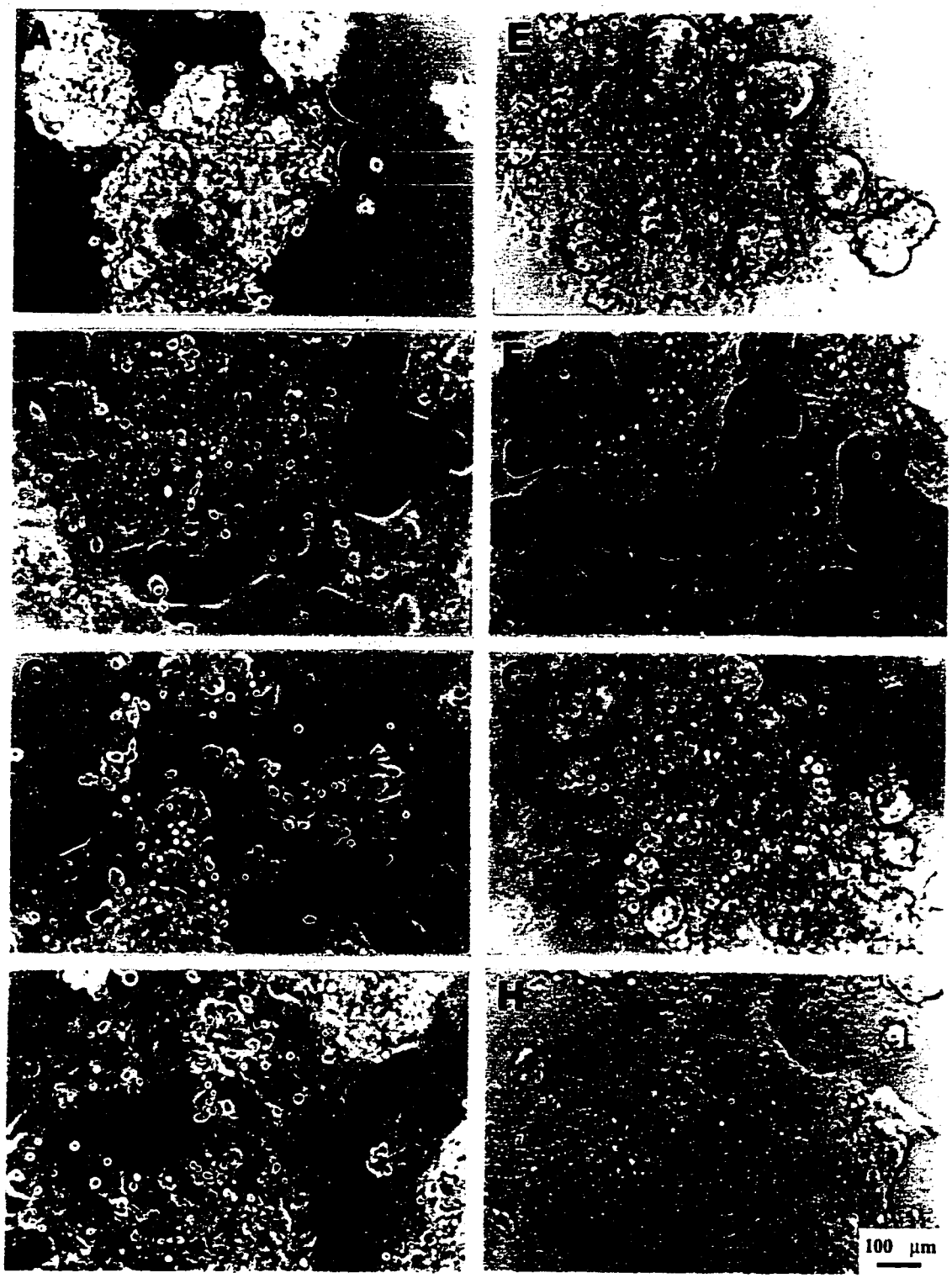
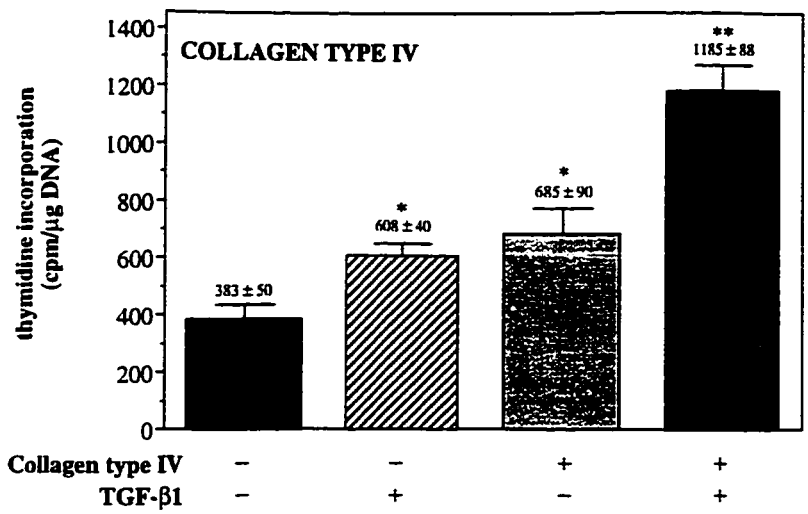
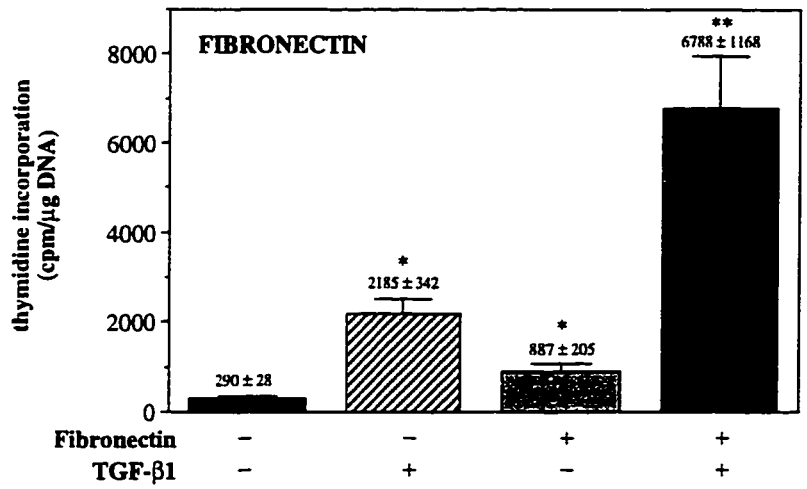
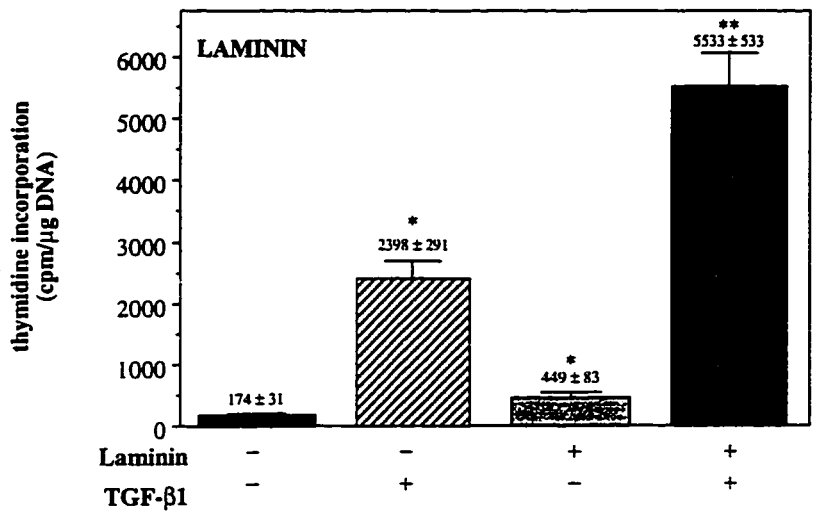

A
E
F
H
100 µm

Figure 15. Effects of ECM substrates on TGF-β1 stimulated CLAP cells: increased thymidine incorporation. CLAP cells were plated directly onto the tissue-culture plastic or on the indicated ECM substrate. CLAP cells were cultured in the presence or absence of 10 pM TGF-β1 for a total of 4 days on collagen and 6 days on fibronectin or laminin. Results are the mean ± S.E. of 18 measurements from 3 experiments. Asterisks indicate significant interactions between treatments. (*) represents $p \leq 0.0001$. (**) represents $p \leq 0.002$. (***) represents $p \leq 0.05$.


COLLAGEN TYPE IV
thymidine incorporation (cpm/µg DNA)
1400
1200
1000
800
600
400
200
0
383 ± 50
608 ± 40
685 ± 90
1185 ± 88
Collagen type IV – – + +
TGF-β1 – + – +


FIBRONECTIN
thymidine incorporation (cpm/µg DNA)
8000
6000
4000
2000
0
290 ± 28
2185 ± 342
887 ± 205
6788 ± 1168
Fibronectin – – + +
TGF-β1 – + – +


LAMININ
thymidine incorporation (cpm/µg DNA)
6000
5000
4000
3000
2000
1000
0
174 ± 31
2398 ± 291
449 ± 83
5533 ± 533
Laminin – – + +
TGF-β1 – + – +

Chapter 4.

The Effects of TGF-β on the Production of Matrix Metalloproteinases.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of enzymes which play an important role in the normal remodeling of extracellular matrices (Matrisian, 1990; Senior and Shapiro, 1992; Mauviel, 1993). All members of this family share common functional structures, including a zinc binding region located at the active site of the enzyme. All members are secreted as inactive proenzymes, resulting from an interaction between the zinc at the active site and a conserved cysteine residue located within the amino-terminal propeptide. Activation can occur by the proteolytic removal of the propeptide or by a disruption of the cysteine-zinc interaction by organomercurial compounds such as amino-phenyl mercurial acetate (APMA). Active MMPs are non-covalently bound by tissue inhibitors of metalloproteinases (TIMPs), which incapacitates the MMPs' proteolytic activity. A subgroup of the MMP family is composed of two gelatinases. The 72 kD gelatinase (MMP2 or type IV collagenase) and the 92 kD (MMP9 or type V collagenase) degrade native collagen types IV, V, VII; denatured collagen (gelatin); and elastin and fibronectin. The promoter regions of MMP2 and MMP9 differ, yet their expression can be coordinately regulated by the multifunctional cytokine, transforming growth factor beta. TGF-β belongs to an expanding superfamily of proteins that regulate an array of cellular responses (Sporn and Roberts, 1992; Attisano, et al., 1994). A major function of TGF-β is the regulation of cell proliferation, but TGF-β also regulates the synthesis and degradation ECM proteins. TGF-β induces the secretion of fibronectin, collagen type IV, laminin, fibronectin and heparan sulfate proteoglycans. TGF-β can modulate the degradation of the ECM by regulating the secretion of MMPs, plasminogen-activators, TIMPs and

plasminogen-activator inhibitors. The specific responses elicited by TGF-β stimulation depend upon the targeted cell type, the cells' interaction with the surrounding extracellular matrix (ECM) and the presence of other regulatory proteins.

The ocular lens continues to grow in size throughout the life of an organism (Persons and Modak, 1970; Kuwabara, 1975). The increase in size is due to the continual addition of lens fiber cells to the periphery of the lens. Lens fibers originate as post-mitotic daughter cells of the germinative zone, a ring of lens epithelial cells located on the anterior surface of the lens. In the avian lens, the post-mitotic daughter cells in the initial stages of terminal differentiation form a structure located on the anterior surface of the lens called the annular pad (Hanna and Keatts, 1966). Annular pad cells migrate posteriorly along the lens capsule towards the lens equator. Once beyond the lens equator, the lens cells rotate their apical-basal axis 180°, elongate tremendously, eliminate all intracellular organelles and become intimately associated with their neighboring lens fiber cells.

Throughout this period of differentiation, the basal surface of the lens cells migrate along the lens capsule. The lens capsule is a continuous basement membrane that surrounds the lens (Kuwabara, 1975; Johnson and Beebe, 1984; Mohan and Spiro, 1986; Webster Jr., et al., 1987). It differs in thickness, composition and architecture between the anterior and the posterior surface. The lens capsule is also bathed in different ocular solutions, the anterior surface contacts the aqueous humor while the posterior side contacts the vitreous humor. Heterogeneity between the different regions of the lens capsule could be the result of soluble matrix-degrading enzymes found within the aqueous or vitreous humors. Alternatively, the differences

found within the lens capsule could result from the preferential production/ modification of the capsule as it is being deposited by the lens cells themselves. Again, the production/modification of the capsule may be under the control of soluble factors within the aqueous or vitreous humors which signal the lens cells to alter their ECM. Regardless of the cause of the differences found within the lens capsule, the function of the heterogeneity could be to direct or sustain cellular migration, provide morphogenetic cues which participate in fiber cell differentiation or serve as sites for the binding and presenting of factors responsible for stimulating lens cell differentiation.

In this study, we explored the possibility that TGF-β may function within the lenticular environment to modify the lens capsule and thereby contribute to its' heterogeneity. This hypothesis is based on two observations. First, TGF-β is potentially available to lens cells *in vivo*. TGF-β has been detected within lens cells and both the aqueous and vitreous humor (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Potts, Bassnett and Beebe, 1995). Secondly, as stated above, several studies have shown that TGF-β modifies the ECM by inducing the expression of MMPs and other enzymes involved in matrix remodeling (Sporn and Roberts, 1992; Attisano, et al., 1994). Thus, the differentiating lens cells could be secreting MMPs in response to TGF-β stimulation. The degradation of the capsule by the MMPs could aide in the migration of the lens cells or function to release matrix-bound growth factors which sustain terminal differentiation. We, therefore, examined the production of MMPs in primary cultures of chicken lens annular pad (CLAP) cells in the presence or absence of TGF-β. We show that the secretion of MMPs is specific to TGF-β

stimulation.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, all reagents were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Tissue culture media and supplements were purchased from GIBCO BRL (Grand Island, NY, U.S.A.) or Celox (Hopkins, MN, U.S.A.). Tissue culture plasticware was purchased from Corning Glass Works (Corning, NY, U.S.A.). Growth factors were purchased from Becton/Dickerson Labware, (Bedford, MA, U.S.A.), except for TGF-β2 which was purchased from Genzyme Corporation (Cambridge, MA, U.S.A.).

Cell Isolation and Culture Treatments. CLAP cells were isolated from 2- to 3-month old chickens as previously described (Ireland, Tran and Mrock, 1993) with minor modifications. In some experiments, 300 μg of total protein were loaded into each well of 24-well tissue culture dishes. Wells contained 1 ml Medium-199 (M199) supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}) and 0.1% fungizone and lacked chicken serum. Cells were treated with TGF-β immediately after being plated and every other day thereafter. Concentrated stocks of a particular growth factor were added directly to the culture medium to yield the indicated dosages. Each experiment was repeated at least three times. Controls received no treatments.

Extracellular Matrix Coatings. In some experiments, prior to the addition of cells, the tissue culture plates were coated with ECM components as previously described (Ireland, Braunsteiner and Mrock, 1993). Laminin, fibronectin or type IV collagen were suspended in sterile phosphate buffered saline (PBS), added to the wells

of 24-well culture dishes and allowed to incubate overnight at 37°C. The final coating concentration for type IV collagen and fibronectin was 1 $\mu g/cm^2$. The final coating concentration for laminin was 10 $\mu g/cm^2$. The solutions were aspirated and the wells washed several times in culture media before the addition of cells.

Detection of matrix metalloproteinases and plasminogen-activators. At the times indicated in the figures, the conditioned culture media was retrieved and cells were harvested. Culture media was concentrated approximately 20-fold by centrifugation in microconcentrators purchased from Amicon inc. (Beverly, MA, U.S.A.). Triplicate aliquots of the concentrated media were taken for determination of total protein concentration. Remaining concentrated media was mixed with equal volumes of 2X zymography sample buffer and frozen at -20°C. Proteinase activity was measured by modification of previously published protocols (Heussen and Dowdle, 1980; Herron, et al., 1986). Samples of concentrated culture media, equalized for total protein, were separated on SDS-PAGE gels copolymerized with gelatin type A. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 minutes with one change of solution. Gels were then incubated overnight at 37°C in 50 mM TRIS, 0.5 mM $CaCl_2$ solution. After incubation, gels were stained in 1% Coomassie Brilliant Blue R, destained and dried. For plasminogen-activator detection, plasminogen was also copolymerized into SDS-PAGE gels containing gelatin. After electrophoresis and exposure to 2.5% Triton X-100, gels were incubated in 0.1 M glycine-NaOH at 37°C for 3-5 hours prior to staining as above.

RESULTS

TGF-β stimulates the production of MMP. Since TGF-β is involved in the

production of both ECM molecules and their degradative enzymes (Sporn and Roberts, 1992; Attisano, et al., 1994), we were interested in the possibility that TGF-β might stimulate cultured CLAP cells to produce MMPs. The secretion of MMPs could enable lens cells to alter their ECM, thereby contributing to the heterogeneity of the lens capsule, assisting in the migration of the differentiating lens cells along the capsule or releasing matrix-bound growth factors. Therefore, we cultured CLAP cells in the absence or presence of 100 or 10 pM TGF-β1. Every 24 hours the culture medium was collected and processed for zymography. From the results shown in figure 16, gelatinase activity is not apparent until 48 hours of culture in the presence of TGF-β. At this time, two bands were detected at molecular weights of approximately 92 kD and 77 kD (arrows). By 72 hours of culture, the production of an additional gelatinase with molecular weight of 72 kD (arrowhead) is evident. At 96 hours of culture and later, the pattern of enzymatic activity remained the same except for the addition of a minor band of approximately 66 kD (circle).

Also evident from figure 16 is that gelatinase activity is only detected in the media collected from TGF-β treated cells, suggesting that the production of gelatinases by CLAP cells is stimulated by TGF-β. The electrophoretic mobilities of the gelatinase activity detected at 72 and 92 kD suggested that the enzymes may be MMP2 and MMP9, respectively. The identity of the 77 kD gelatinase is unknown. Gelatinase activity was inhibited by the inclusion of 10 mM EDTA in the incubation buffer. The lack of activity in the presence of a metal chelator confirms that the gelatinase bands are products of metalloproteinase activities. Identical results were obtained with TGF-β1 or TGF-β2 stimulation.

Modification of MMPs. Because the pattern of gelatinase activity was modified as the time in culture increased, it was of interest to investigate the activation of MMPs. MMPs can be activated *in vitro* by incubating the enzymes in the presence of organomercurials such as APMA (Springman, et al., 1990; Woessner Jr., 1991). APMA interferes with the zinc-cysteine interaction at the active site of the enzyme. Destabilizing the interaction results in the autocatalytic removal of the propeptide and the appearance of gelatinase activity at a slightly lower molecular weight. Therefore, we cultured CLAP cells in the absence or presence of 100 or 10 pM TGF-β1 for 4 days. Culture medium was concentrated and either immediately mixed with zymography sample buffer or incubated at room temperature for an additional 18 hours in the absence or presence of 1 mM APMA prior to the addition of sample buffer. The zymogram of such an experiment is shown in figure 17A. Culture media mixed immediately with zymography sample buffer exhibited the same pattern of gelatinase activity as untreated culture media that remained at room temperature for an additional 18 hours. APMA treatment of the culture media for 18 hours resulted in the alteration of the TGF-β stimulated pattern of gelatinase activity (arrows). Figure 17B is a zymogram of the culture media from CLAP cells grown in the presence of 100 pM TGF-β for 7 days. The concentrated media was incubated for an additional 5 hours in the presence or absence of 1 mM APMA. APMA exposure of the culture medium from TGF-β treated cells resulted in the activation of MMP2, as evident by the appearance of a gelatinase band at approximately 66 kD. This is similar to the observed modification of MMP2 at 96 hours of TGF-β stimulation (figure 16). APMA exposure did not result in the activation of the gelatinase believed to be MMP9

nor the unknown 77 kD gelatinase, but caused the disappearance of the activities of both gelatinases. Media treated with APMA also contained a new band of gelatinase activity at a slightly higher molecular weight than 92 kD.

TGF-β stimulates MMP activity associated with CLAP cell membranes. All MMPs, except MMP2, can be activated *in vivo* by the serine proteinase plasmin. It was recently discovered that MMP2 is activated by a new group of membrane associated MMPs called MT-MMPs or membrane-type MMPs (Murphy and Docherty, 1992). Since activated MMP2 was detected in the culture medium of CLAP cells exposed to TGF-β for 96 hours, we examined the MMP activity associated with CLAP cell membranes. CLAP cells were cultured in the absence or presence of 100 or 10 pM TGF-β1 for 5 days. Cells were collected, briefly sonicated and centrifuged. The resulting pellet of cell membranes was dissolved in sample buffer and subjected to zymographic electrophoresis. MMP activity associated with CLAP cell membranes was detected at approximately 77 kD only in TGF-β treated cells (figure 18).

TGF-β stimulates the secretion of a plasminogen-activator-like protein. Plasmin is produced from its latent form, plasminogen by enzymes known as plasminogen-activators (Vassalli, Sappino and Belin, 1991). Because plasmin can function to activate latent proMMPs as well as latent TGF-β, it plays a pivotal role in the maintenance or degradation of the ECM. Thus, we examined the production of plasminogen-activators (PAs) by CLAP cells cultured in the presence or absence of 100 pM TGF-β1 or TGF-β2. From figure 19, it is apparent that TGF-β also stimulates the secretion of PA activity at the approximate molecular weights of 40-45 kD (arrows), which lie within the reported range of molecular weights for urokinase

(uPA).

Effects of an ECM on MMP secretion. CLAP cells cultured in the presence of TGF-β attach and spread out onto the tissue culture vessel in a dose-dependent manner (see Chapter 2). It was of interest to determine if MMP production was specific to TGF-β stimulation and not just a general response to cellular spreading. Therefore, we cultured CLAP cells on uncoated tissue culture plastic or on plastic coated with collagen type IV, fibronectin or laminin. CLAP cells cultured on an ECM substrate display enhanced attachment and spreading, similar to that seen with TGF-β (Ireland, Braunsteiner and Mrock, 1993). Cells cultured on ECM substrates in the presence of 10 pM TGF-β exhibit monolayer growth. Zymograms of the culture media from the above conditions are shown in figure 20. MMP activity is detected only in the culture media collected from TGF-β treated cells, independent of the type of ECM the cells were grown on. These results suggests that MMP production is specific to TGF-β stimulation and is not a response to cellular spreading.

Additional growth factors stimulate the secretion of MMPs. We next wanted to determine if the observed pattern of MMP activity was induced specifically by TGF-β or if MMP production was a non-specific response of cultured CLAP cells to growth factor stimulation. CLAP cells were cultured in the presence or absence of TGF-β, EGF, FGF, IGF-1 or PDGF. Concentrations of the growth factors varied as we tried to equalize the degree of cellular spreading onto the culture vessel. The zymogram of the culture media from CLAP cells treated with the different growth factors revealed varied patterns of growth factor induced MMP secretion (figure 21). TGF-β stimulation evoked the secretion of MMP2, MMP9 and the unknown 77 kD gelatinase.

Exposure to EGF or IGF-1 elicited the production of MMP9 and MMP2, respectively. PDGF stimulated the secretion of both MMP2 and MMP9. No MMP activity was detected in the culture media of FGF treated cells. EGF, IGF-1 and PDGF induced the production of MMPs, but not to the same extent nor with the specific pattern that is observed with TGF-β stimulation.

DISCUSSION

Gelatinase production and activation. The heterogeneity of the lens capsule could function to guide cellular migration, sustain fiber cell differentiation or present the specific factors responsible for stimulating differentiation. The differences between the various regions of the lens capsule may result from preferential production or degradation/modification of certain ECM molecules. The specific proteinases responsible for altering the lens capsule could be secreted by the lens cells themselves. We hypothesized that TGF-β may function within the lenticular environment to stimulate the secretion of gelatinases, resulting in the modification of the lens capsule.

The data presented in this report reveal that CLAP cells cultured in the presence of TGF-β secrete gelatinases with the molecular weights of 72, 77 and 92 kD. Gelatinase activity at the approximate molecular weights of 92 and 77 kD was first detected at 48 hours of culture. With continued culture, gelatinase activity at approximately 72 kD becomes evident. Based on the electrophoretic mobilities, the gelatinase activity detected at 72 and 92 kD suggests that these enzymes may be MMP2 and MMP9, respectively. The identity of the 77 kD gelatinase is unknown. A fourth gelatinase with the approximate molecular weight of 66 kD can be detected by 96 hours of culture. The 66 kD gelatinase may be the activated form of MMP2.

Gelatinase activity was only detected in media collected from cells cultured in the presence of TGF-β, suggesting that the production of gelatinases by CLAP cells is stimulated by TGF-β. Thus, TGF-β induces cultured CLAP cells to secrete an unknown 77 kD gelatinase and two gelatinases similar to MMP2 and MMP9.

Brown, et al. (1994) reports that gelatinase activity is absent from extracts of human lenses. This agrees with our observation that enzyme activity was never detected in the absence of TGF-β, yet conflicts with our hypothesis that TGF-β is functioning *in vivo* to modify the lens capsule by stimulating the production of MMPs. A possible explanation of this discrepancy could be that the normal levels of lenticular gelatinases are undetectable by zymography. If the gelatinases are functioning to assist with the migration of the differentiating lens cells or to release matrix-bound growth factors, the region of gelatinase activity is very localized. It is likely that the amount of enzyme contained within an aliquot of whole lens extract could not be detected by zymography.

The data presented here support other studies demonstrating that TGF-β induces epithelial cells to secrete both MMP2 and MMP9. Experiments performed by Salo, et al. (1991) showed that TGF-β induced human mucosal and epidermal keratinocytes to consistently express MMP9 at greater levels than the expression of MMP2. Stampfer, et al. (1993) also reports the coordinated induction of both gelatinases by TGF-β in human mammary epithelial cell lines, but with the induction of MMP2 expression being greater than that of MMP9. In that study, TGF-β also slightly increased the activities of a 62 and an 82 kD gelatinase, the activated forms of MMP2 and MMP9, respectively. Our results reveal that CLAP cells stimulated by TGF-β secrete

equivalent amounts of both gelatinases as well as an additional gelatinase with the molecular weight of 77 kD.

All MMPs can be activated *in vitro* by organomercurial compounds such as APMA (Springman, et al., 1990; Woessner Jr., 1991). APMA activates proMMPs by perturbing the cysteine-zinc interaction at the active site of the enzyme. Disrupting the interaction results in the autocatalytic removal of the propeptide and the appearance of gelatinase activity at a slightly lower molecular weight. APMA treatment of culture media collected from TGF-β stimulated cells resulted in the appearance of a band of activity located below MMP2 at approximately 66 kD. Since gelatinase activity at 72 kD is still evident, APMA treatment only partially converted MMP2 to its active form. MMP2 is activated *in vivo* by membrane type-matrix metalloproteinases (MT-MMPs) located within the cell membrane. Therefore, we examined the gelatinolytic activity of crude membrane preparations from TGF-β stimulated CLAP cells. Zymograms revealed a gelatinase band at approximately 77 kD. The reported molecular weights for human MT-MMPs are approximately 65 kD (Sato, et al., 1994; Strongin, et al., 1995). Differences in molecular weights may reflect variations between the species. Thus, the gelatinase activity detected in the crude membrane preparations of CLAP cells may represent a chicken MT-MMP or a newly identified secreted gelatinase which remains matrix-associated.

As stated above, APMA treatment should normally cause the appearance of gelatinase activity at slightly lower molecular weights than the latent proforms. APMA treatment of culture media collected from CLAP cells stimulated by TGF-β unexpectedly resulted in the disappearance of both the 92 and the 77 kD gelatinase

with the simultaneous appearance of a band of activity located slightly higher than 92 kD. This observation remains unexplainable. Although the authors do not discuss it within the text of their report, Brown, et al. (1990) show the disappearance of MMP9 in APMA treated samples of conditioned culture medium from HT-1080 cells.

Both TGF-β and MMPs can be activated *in vivo* by the serine proteinase plasmin (Vassalli, Sappino and Belin, 1991). Plasmin is produced by the proteolytic cleavage of plasminogen by the enzymes, plasminogen-activators (PAs). Data presented here demonstrates that TGF-β can stimulate CLAP cells to secrete proteins resembling uPA. The presence of uPA within the lenticular environment provides a key element in the integrated pathways possibly involved with the modification of the lens capsule. uPA cleaves plasminogen to form plasmin. Plasmin can activate proMMPs and latent TGF-β. Active TGF-β, in turn, stimulates the production of additional uPAs and induces the secretion of proMMPs. Active MMPs then function to modify the lens capsule which may assist lens cell migration and affect fiber cell differentiation. Therefore, uPA is an important component of TGF-β's matrix modifying activity.

In support of the idea that TGF-β may affect lens cell migration and differentiation by modifying the lens capsule are studies examining the localization of uPA (Tripathi, Tripathi and Park, 1990). Using immunocytochemistry, uPA was identified in the posterior lens capsule and in the equatorial epithelial cells, but could not be detected in the anterior capsule nor the central epithelium. This distribution of uPA reflects functional differences in cellular motility between the different regions of the lens. Central epithelial cells are stationary and remain attached to the anterior lens

capsule, therefore, uPA would not be expected to be expressed. Localization of uPA to the posterior capsule and equatorial epithelial cells suggests that these cells may be using the plasmin and MMP systems to produce a matrix capable of directing their migration. Since uPA synthesis may be controlled by TGF-β stimulation, this suggests that one possible function of TGF-β in the lens is establishing the highly localized expression of uPA.

Specificity of gelatinase production. TGF-β stimulated the secretion of gelatinase activity at approximately 92, 77 and 72 kD. Gelatinase activity was never detected in media collected from CLAP cells cultured in the absence of TGF-β. TGF-β also stimulated, in a dose-dependent manner, enhanced cellular attachment and spreading of CLAP cells onto the culture vessel. To be assured that gelatinase production was stimulated by TGF-β and not the result of cellular attachment and spreading, we grew CLAP cells on ECM-coated tissue-culture plastic. CLAP cells grown on an ECM substrate display similar amounts of attachment and spreading to cells cultured in the presence of TGF-β. Zymograms of media from CLAP cells cultured on the different ECM substrates in the presence or absence of TGF-β revealed that the presence of ECM components and cellular spreading does not induce gelatinase production. The detected pattern of enzymatic activity is specific to TGF-β stimulation. Again, our results are in agreement with the data reported by Salo, et al. (1991). Human keratinocytes were cultured on substrates of fibronectin, collagen type I or laminin, in the absence or presence of TGF-β. Expression of MMP2 and MMP9 was induced by TGF-β stimulation, independent of the type of ECM the cells were grown on.

To determine if the pattern of enzymatic activity detected in the media collected from TGF-β treated cells is specific to TGF-β, CLAP cells were cultured in the presence of other growth factors. Culture media collected from CLAP cells stimulated with IGF-1, EGF, FGF or PDGF revealed some gelatinase activity, but to a lesser extent than the activity observed with TGF-β stimulation. No gelatinolytic activity was observed in the media collected from CLAP cells cultured in the presence of FGF. The secretion of all three gelatinases is specific to TGF-β stimulation.

The production of MMPs in response to stimulation by a specific growth factor has not been studied extensively. In agreement with our results, EGF has been reported to stimulate the secretion of MMP9 (Lyons, et al., 1993; Shima, et al., 1993). bFGF-induced invasiveness of bovine capillary endothelial cells can be inhibited by the presence of anti-gelatinase antibodies (Mignatti, et al., 1989). This suggests that bFGF is stimulating the secretion of MMPs which permit the endothelial cells to become invasive. Contrary to this report, Unemori, et al. (1991) reports that bFGF has no effect on the production of MMP9. Our results also imply that FGF does not induce the production of MMP9, nor MMP2.

In summary, TGF-β specifically stimulates CLAP cells to secrete gelatinases. TGF-β can also stimulate the production of uPA, thereby providing a mechanism in which to activate the proMMPs and additional latent TGF-β. The production of MMPs within the lenticular environment may function to modify the lens capsule in order to assist cellular migration, provide morphogenetic cues which participate in fiber cell differentiation or release matrix-bound factors responsible for stimulating lens cell differentiation.

TGF-β stimulation of epithelial cells also induces the production of plasminogen-activator inhibitors and tissue inhibitors of metalloproteinases (Attisano, et al., 1994; Gerwin, et al., 1990; Sporn and Roberts, 1992; Stampfer, et al., 1993). Production of the specific inhibitors may serve to localize enzymatic activity to the leading edge of the migrating lens cell or to regulate the degree of capsular degradation/modification. Future studies should provide information on the regulation of MMP activity and the mechanisms directing lens cell migration.

Figure 16. Time course of TGF-β stimulated MMP secretion. Zymograms of culture media from CLAP cells grown in the presence or absence of TGF-β for the times indicated. (St) Culture media from human fibrosarcoma cells (HT 1080) known to secrete both 72 and 92 kD gelatinases. Media from control (C) cultures and cells cultured in the presence of TGF-β at the indicated picomolar concentrations. Arrows indicate gelatinase activity at approximately 92 and 77 kD. Note the increase in activity during longer times in culture. Open arrowheads indicate the location of the 72 kD gelatinase. Circle indicates the position of the active 66 kD form of the 72 kD gelatinase.

TIME COURSE OF MMP PRODUCTION

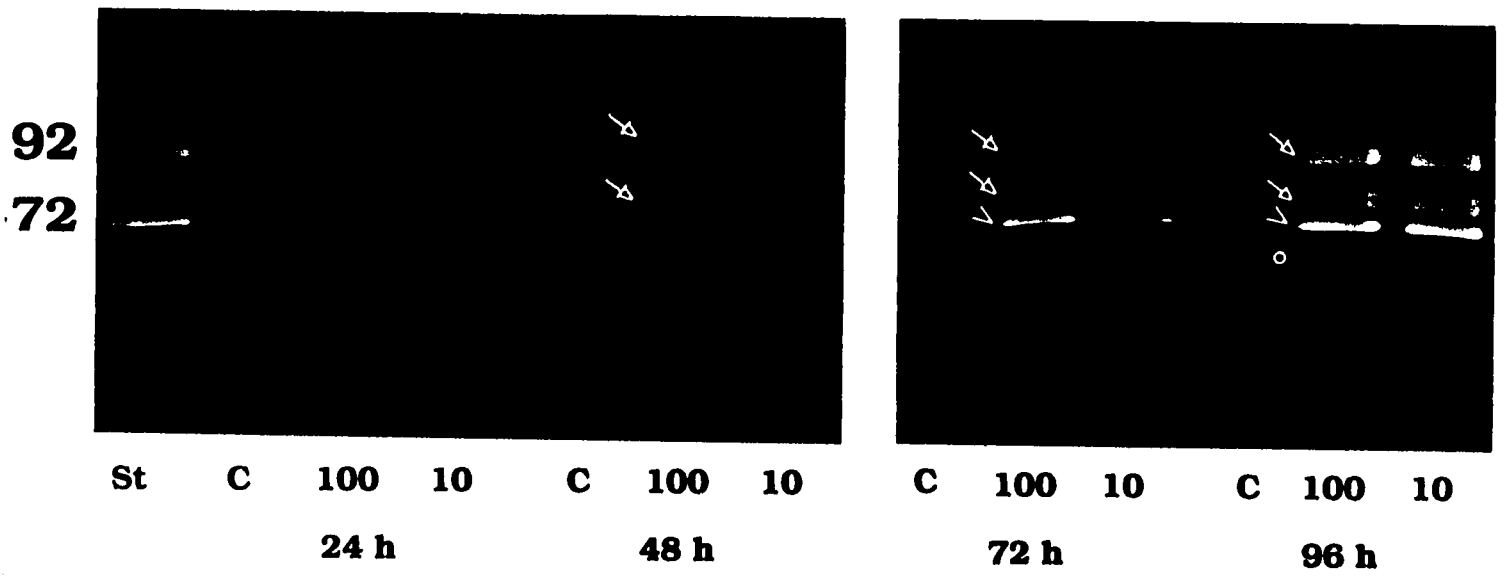
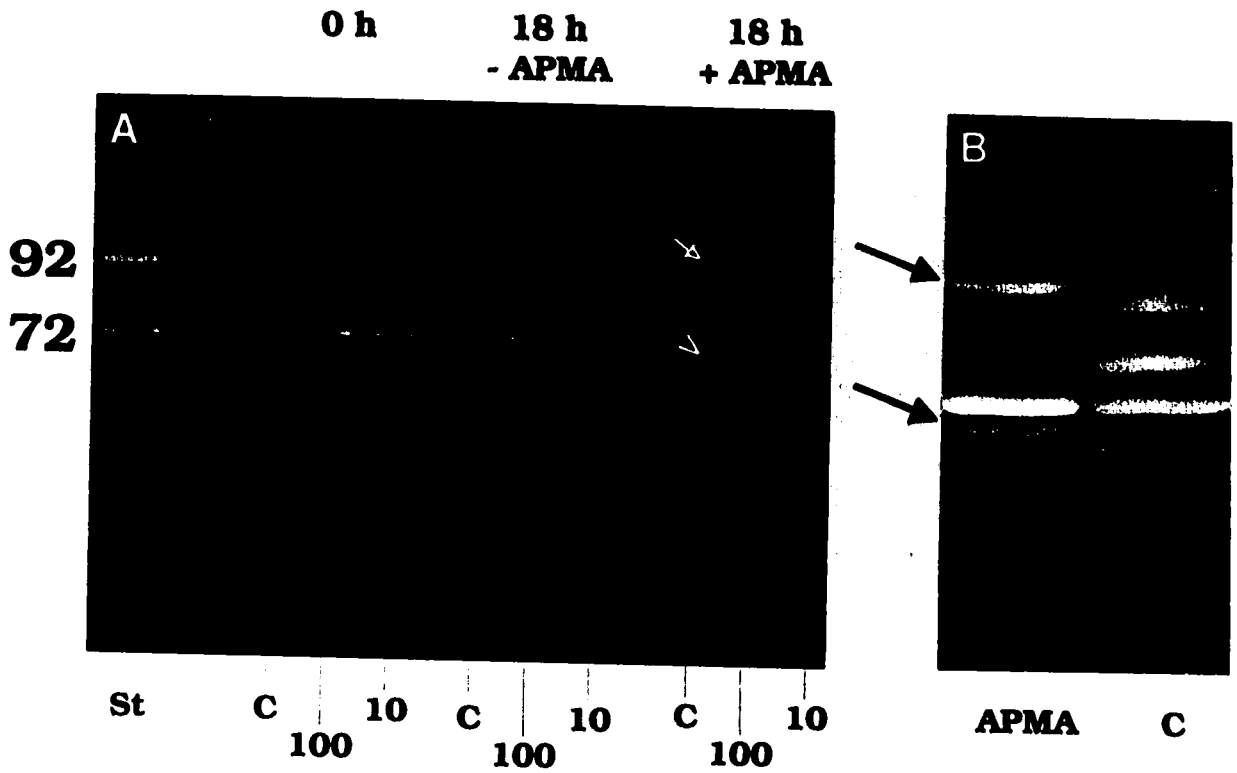


Figure 17. APMA modification of TGF-β stimulated MMP. Panel A: Zymogram of culture media from CLAP cells grown in the presence or absence of TGF-β for 4 days. Concentrated media was either mixed immediately with zymography sample buffer (0 h) or incubated at room temperature for an additional 18 hours in the absence (18 h-APMA) or presence (18h+APMA) of 1 mM APMA prior to the addition of sample buffer. (St) Culture media from human fibrosarcoma cells (HT 1080) known to secrete both 72 and 92 kD gelatinases. Media from control (C) cultures and cells cultured in the presence of TGF-β at the indicated picomolar concentrations. Arrows indicate gelatinase activity in APMA-treated samples which differ in molecular weight from non-treated samples.

Panel B: Zymogram of culture media from CLAP cells grown for 7 days in the presence of 100 pM TGF-β. Concentrated culture media was incubated at room temperature for an additional 5 hours in the presence (APMA) or absence (C) of 1 mM APMA. Arrows indicate gelatinase activity in APMA-treated samples which differ in molecular weight from non-treated samples. Note the disappearance of both the 92 and the 77 kD gelatinase in the APMA treated samples.

MODIFICATION OF LATENT MMPs



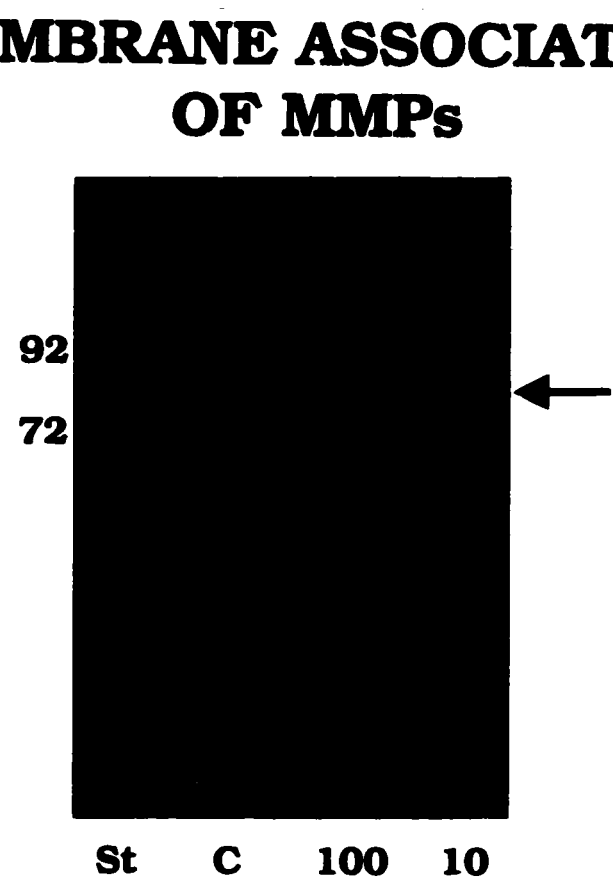


Figure 18. TGF-β stimulates MMP activity associated with CLAP cell membranes. Zymograms of whole cell lysates of CLAP cells cultured in the presence or absence of TGF-β for 5 days. (St) Culture media from human fibrosarcoma cells (HT 1080) known to secrete both 72 and 92 kD gelatinases. Whole cell lysates of control (C) cultures and cells cultured in the presence of TGF-β at the indicated picomolar concentrations. Arrow indicates location of MMP activity associated with CLAP cell membranes at approximately 77 kD.

EFFECTS OF TGF-β ON THE PRODUCTION OF A uPA-LIKE PROTEIN

Figure 19. TGF-β stimulates the secretion of a uPA-like protein. Plasminogen containing zymogram of culture media from CLAP cells grown in the presence or absence of TGF-β for 48 hours. (MW) Molecular weight standards in kilodaltons. (El) and (Tr) Elastase and trypsin standards, respectively. Media from control (C) cultures and cells cultured in the presence of 100 pM TGF-β1 (B1) or TGF-β2 (B2). Arrows indicate activity of plasminogen-activator-like proteases at approximately 40-45 kD.

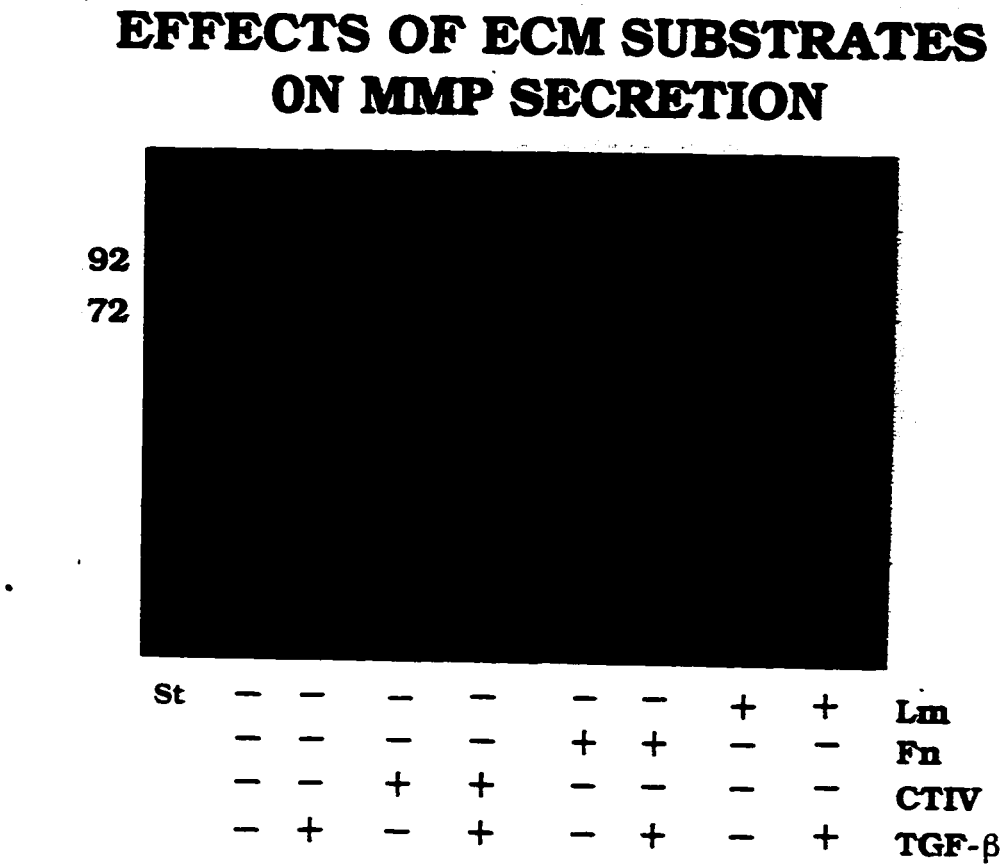


Figure 20. Effects of ECM substrates on TGF-β stimulated MMP secretion. Zymograms of culture media from CLAP cells grown on an ECM substrate in the presence or absence of 10 pM TGF-β1. Cells were plated onto uncoated tissue-culture plastic or plastic coated with collagen type IV (CTIV), fibronectin (Fn) or laminin (Lm). (St) Culture media from human fibrosarcoma cells (HT 1080) known to secrete both 72 and 92 kD gelatinases.

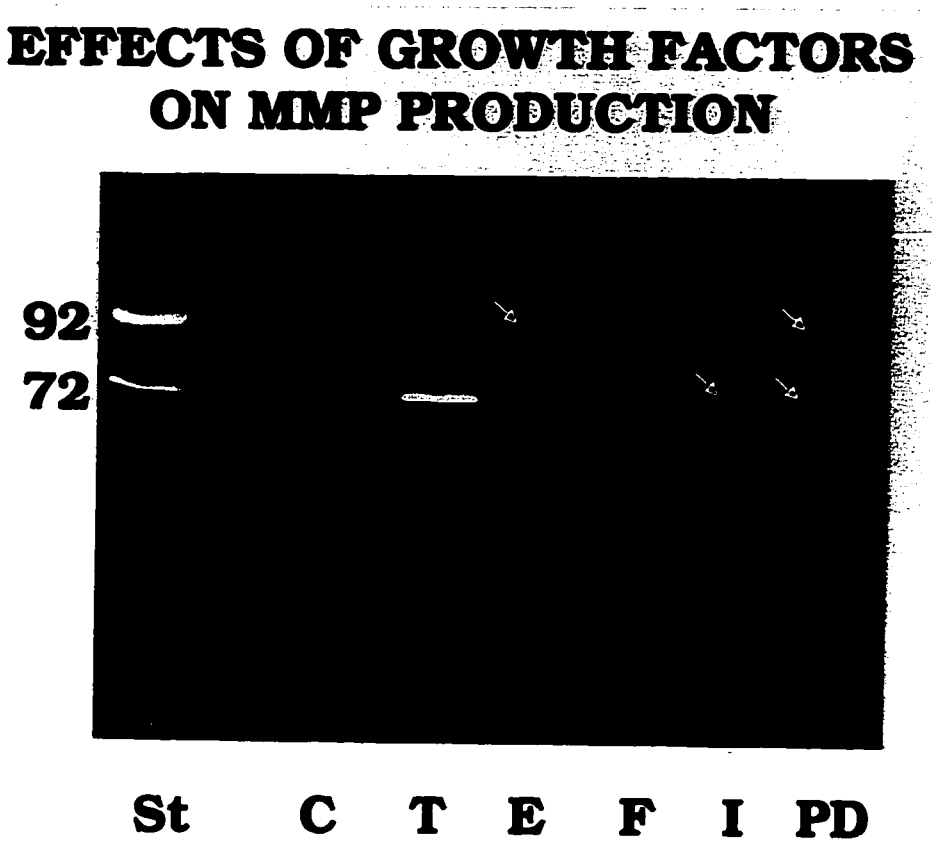


Figure 21. Effects of growth factors on MMP secretion. Zymogram of culture media from CLAP cells grown for 7 days in the absence (C=media from control cultures) or presence of (T) 2.5 ng/ml TGF-β, (E) 100 ng/ml EGF, (F) 100 ng/ml FGF, (I) 10 ng/ml IGF or (PD) 10 ng/ml PDGF. (St) Culture media from human fibrosarcoma cells (HT 1080) known to secrete both 72 and 92 kD gelatinases. Arrows indicate MMP activity associated with EGF, IGF and PDGF treatment.

Chapter 5.

Conclusions

CONCLUSIONS

The aim of this study was to investigate the functions of TGF-β in the ocular lens. The lens is comprised of concentric shells of lens fiber cells overlaid by an anterior single layer of lens epithelial cells. Lens cells destined to become lens fiber cells originate from the periphery of the anterior lens epithelium. These cells undergo a final mitotic division and begin the process of terminal differentiation as they migrate posteriorly along the lens capsule, the continuous basement membrane surrounding the lens. Terminal differentiation involves permanent withdrawal from the cell cycle; cellular elongation; loss of intracellular organelles and the increased synthesis of lens-specific crystallins, cytoskeletal and membrane proteins (Persons and Modak, 1970; Kuwabara, 1975).

TGF-β has been located within both the aqueous and vitreous humors as well as within lens cells themselves (Jampel, et al., 1990; Cousins, et al., 1991; Lutty, et al., 1993; Potts, Bassnett and Beebe, 1995). TGF-β has the ability to bind heparan sulfate proteoglycans, fibronectin and type IV collagen, suggesting that it could also be a component of the lens capsule (Fava and McLure, 1987; Boyd, et al., 1990; Paralkar, Vukicevic and Reddi, 1991; Attisano, et al., 1994). Several studies have shown that TGF-β can inhibit the proliferation of epithelial cells, including the lens epithelium (Sporn and Roberts, 1992; Attisano, et al., 1994; Kurosaka and Nagamoto, 1994; Nishi, et al., 1996). We therefore explored the possibility that TGF-β may influence terminal differentiation of fiber cells by maintaining them in a permanently post-mitotic state or by promoting the appearance of differentiated characteristics.

We began this study by demonstrating that CLAP cells expressed both type I

and type II TGF-β receptors. Co-expression of both receptor types indicates that CLAP cells are capable of detecting and transducing a TGF-β signal intracellularly. We were unable to detect the expression of TGF-β receptors in superficial lens fiber cells. These experiments are in agreement with the immunohistochemical studies performed by Obata, et al. (1996). They also report the presence of both TGF-β receptors in rat lens epithelial cells, but not in the fiber cells. It is believed that lens fiber cells are not lacking TGF-β receptors, but that the expression of both receptors within the fiber cells is too low to be detected.

We then started to characterize some of the *in vitro* responses of cultured CLAP cells to TGF-β stimulation. TGF-β stimulation, either in the presence or absence of serum, did not inhibit the proliferation of cultured CLAP cells. Instead, TGF-β stimulated dose-dependent increases in both thymidine incorporation and cellular attachment and spreading. Characteristics of fiber cell differentiation were not enhanced by TGF-β stimulation. Exposure to TGF-β caused a decrease in the synthesis of lens-specific crystallins and no accumulation of phakinin, a specific marker protein for lens cell differentiation. Thus, TGF-β stimulation of cultured CLAP cells does not inhibit mitosis nor does it lead to an increase in differentiated characteristics.

The observed responses of cultured CLAP cells to TGF-β stimulation may not reflect those elicited *in vivo*. Cells placed into a culture system are removed from a complex environment. Conditions which would normally inhibit mitosis, modulate the effects of various stimuli and promote the acquisition of further differentiated traits are absent. Therefore, the results obtained from experiments reported here must be

interpreted while remembering that the elicited *in vivo* responses are the combined result of an integrated framework of multiple growth factors and hormones.

As the terminally differentiating lens cells migrate along the lens capsule, they encounter differences within their microenvironments. The lens capsule differs in thickness, composition and architecture between the anterior and the posterior surfaces (Kuwabara, 1975; Johnson and Beebe, 1984; Mohan and Spiro, 1986; Webster Jr.,1987). The lens capsule is also in contact with different ocular solutions. The anterior surface is bathed in aqueous humor, while the posterior side is covered with vitreous humor. The aqueous and vitreous humors differ in their ability to influence lens cell behavior (Coulombre and Coulombre, 1963; Lovicu, Chamberlain and McAvoy, 1995). Thus, the migrating lens cells encounter a varying extracellular matrix and dissimilar soluble factors. Differences within the microenvironments may affect the morphology and biochemical behavior of the differentiating lens cells.

TGF-β functions not only to regulate cell proliferation and differentiation, but also to influence the secretion of ECM proteins and their degrading proteinases (Sporn and Roberts, 1992; Attisano, et al., 1994). TGF-β stimulation may also regulate the selective expression of specific integrin subunits (Ignotz and Massagué, 1987; Heino, et al., 1989). The specific combination of the various integrin subunits determines the specificity of the interactions between the cell and particular components of the ECM. Therefore, TGF-β has the ability to affect cell functioning both directly and indirectly. TGF-β indirectly regulates cellular behavior through the modification of the ECM and by the alteration of a cell's ability to interact with the ECM constituents.

Studies have demonstrated that TGF-β stimulation of lens cells results in the

accumulation of ECM molecules (Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995), suggesting that TGF-β functions in the lens to stimulate the production of ECM components. The TGF-β induced production of matrix components could alter the lens capsule which in turn could modify lens cell behavior. Studies performed in our laboratory have shown that cultured CLAP cells presented with an ECM substratum respond with increased levels of both thymidine incorporation and cellular spreading (Ireland, Braunsteiner and Mrock, 1993). Therefore, we examined 1) if TGF-β could affect the production of lens capsule components by cultured CLAP cells and 2) if the presence of an ECM substratum could affect TGF-β induced responses of cultured CLAP cells.

Our results show that CLAP cells stimulated by TGF-β secrete collagen type IV and fibronectin into the culture medium and deposit a matrix onto the bottom of the culture dish that differs from the matrix produced by untreated cells. Although, laminin is an abundant capsular glycoprotein, the production of laminin was never observed. The observation that TGF-β stimulates the production of fibronectin by cultured CLAP cells was of interest, since fibronectin is the least abundant protein found within the bovine lens capsule (Cammarata and Spiro, 1985) and has been reported to be completely absent from adult rat capsules (Parmigiani and McAvoy, 1991). The TGF-β induced synthesis of fibronectin could function to influence the motility of the differentiating lens cells. Fibronectin has been shown to enhance the migratory potential of epithelial cells (O'Keefe, et al., 1985; Nickoloff, et al., 1988; Olivero and Furcht, 1993). These studies are in contrast with the results obtained by Parmigiani and McAvoy (1991). They report that epithelial cells obtained from

explants of embryonic and neonatal rat lenses lose their ability to migrate onto a fibronectin substrate as the animal ages; while cells of all ages retain the ability to migrate onto laminin. The conflicting results on the role of fibronectin in cell migration implies that multiple ECM molecules are involved with the *in vivo* migration of lens cells. Although fibronectin comprises only a small portion of the capsular constituents, the concentration of the protein is highest in the posterior capsule (Cammarata and Spiro, 1985; Mohan and Spiro, 1986). It is possible that the TGF-β induced production of fibronectin may slightly alter the composition of the posterior lens capsule and thereby enhance the migration of the lens fiber cells.

As mentioned above, TGF-β is found within both the aqueous and the vitreous humors, yet the concentration of fibronectin is highest in the posterior capsule. This may be explained by the observation that the cells' state of differentiation has been shown to alter the responses elicited by TGF-β stimulation. Proliferating astrocytes secrete fibronectin in response to TGF-β. Once the astrocytes have begun to differentiate, they become refractory to TGF-β stimulation (Toru-Delbauffe, et al., 1992). In another study, TGF-β stimulation of undifferentiated keratinocytes induced an increase in the gene expression of fibronectin, laminin and collagen type IV. In contrast, TGF-β treatment of differentiated keratinocytes stimulated an increase in the expression of only fibronectin (Salo, et al., 1991). These experiments suggest that the state of differentiation can change the induction of specific ECM genes by TGF-β. Therefore, TGF-β stimulation of differentiating lens fiber cells may preferentially be eliciting the production of fibronectin over the other constituents of the lens capsule. The synthesis of different basement membrane components induced by TGF-β alters

the composition and structure of the matrix. Any changes in the ECM may have lasting effects on the physiology of the associated cells.

TGF-β stimulation also induced the production of an unidentified protein with extremely high molecular weight, which may represent HSPG. TGF-β has been reported to stimulate the production of HSPGs by rat lens epithelium (Liu, et al., 1994; Hales, Chamberlain and McAvoy, 1995). The posterior lens capsule contains a greater concentration, and a larger species, of HSPG than the anterior lens capsule (Mohan and Spiro, 1986). It has been shown that TGF-β stimulates the production of HSPGs with higher molecular weights than the HSPGs produced by untreated fibroblasts (Nugent and Edelman, 1992). Thus, the preferential production of larger HSPGs in the posterior capsule could result from TGF-β stimulation of terminally differentiating lens epithelial cells.

HSPG synthesis in the lens is important because of its intimate relationship with bFGF. HSPG can potentiate the activity of bFGF, possibly by presenting bFGF to high affinity cell surface receptors (Nugent and Edelman, 1992; Cook, Doumit and Merkel, 1993; Falcone, et al., 1993). The high affinity binding of bFGF for HSPG may also serve to protect bFGF from degradation. Immunohistochemical studies performed by Lovicu and McAvoy (1993) demonstrated the co-localization of bFGF and HSPG within rat lens capsules. In studies performed with fibroblasts and adrenocortical cells, the TGF-β induced production of HSPGs correlated with enhanced binding of bFGF and an increase in the mitogenic activity of bFGF (Jiang, et al., 1992; Nugent and Edelman, 1992). The TGF-β induced production of larger HSPGs in the posterior capsule suggests that a higher concentration of bFGF would be bound

to the posterior surface than to the anterior capsule. In fact, sections of embryonic mouse eye showed the posterior surface of the capsule bound more bFGF than the anterior surface (Jeanny, et al., 1987).

Several studies report the importance of FGF in lens fiber cell differentiation. Both aFGF and bFGF stimulate proliferation and fiber differentiation in subcultures of human lens epithelium (Ibaraki, Lin and Reddy, 1995). Cell elongation, organelle loss, interdigitations of lateral cell membranes and the accumulation of β-crystallins all can be induced by bFGF treatment of rat epithelial explants (Lovicu and McAvoy, 1989; McAvoy and Chamberlain, 1989). IGF-1 has been shown to enhance bFGF-induced fiber differentiation (Richardson, Chamberlain and McAvoy, 1993). Thus, the TGF-β induced modification of the lens capsule may alter the ECM binding of growth factors which could moderate the growth factor's activity and ultimately, regulate lens cell behavior.

So far, we have shown that TGF-β is capable of stimulating cultured CLAP cells to produce ECM molecules which may subtly change the lens capsule and potentially modify the behavior of lens cells. Therefore, it may be possible that the enhanced accumulation of ECM induced by TGF-β is responsible for stimulating the increased thymidine incorporation of cultured CLAP cells that was seen in Chapter 2. Previous studies performed in our laboratory have shown that CLAP cells presented with an ECM substratum exhibit increased levels of both thymidine incorporation and cellular spreading (Ireland, Braunsteiner and Mrock, 1993). Other studies have shown that cellular flattening and spreading onto a substrate serves as a strong mitotic stimulus to lens epithelial cells (Glaesser, Rattke and Iwig, 1979; Iwig, Glaesser and

Bethge, 1981). Thus, the proliferation of CLAP cells cultured in the presence of TGF-β may reflect a secondary response to the accumulation of ECM stimulated by TGF-β; and TGF-β could still function *in vivo* to inhibit the proliferation of differentiating lens cells.

TGF-β may function *in vivo* to modify the lens capsule which may facilitate migration or sustain terminal differentiation. In turn, the lens capsule could be influencing the behavior of the lens cells by modulating their responses to specific signaling factors like TGF-β. Therefore, we examined the effects the presence of an ECM substratum could have on TGF-β induced responses of cultured CLAP cells. CLAP cells cultured on an ECM substratum in the presence of TGF-β displayed monolayer growth and synergistic increases in thymidine incorporation, indicating that the responses of CLAP cells to TGF-β can be modulated by the extracellular environment.

Simultaneous stimulation of CLAP cells by both TGF-β and an ECM substratum resulted in synergistic responses. The heightened responses may be the result of the ECM constituent presenting the TGF-β molecule in the proper orientation to TGF-β receptors or the result of an unknown interaction between the two signaling pathways. A possible common link to both signaling pathways are the integrins. Since integrins couple the extracellular environment to the cytoskeleton of the cell, they not only function to attach a cell to its matrix, but also to activate signaling cascades which interact with growth factor signaling pathways (Hynes, 1992; Schlaepfer, et al., 1994; Sastry, et al., 1996). Through the interaction of signaling pathways, integrins can modulate cellular responses which were initially elicited by

extracellular growth factors.

Individual integrins are heterodimers of covalently linked α and β subunits. It has been shown that both the nature of the ECM and TGF-β can individually regulate the expression of integrin subunits (Heino and Massagué, 1989; Heino, et al., 1989; Delcommenne and Streuli, 1995). Although not examined in this report, the presence of a ECM substratum or stimulation by TGF-β could also modulate CLAP cell expression of integrins. Integrins could then modify cell behavior by enhancing cell division. Therefore, the synergistic increase in thymidine incorporation and the monolayer growth observed in CLAP cells cultured on an ECM substrate in the presence of TGF-β may result from a combination of 1) direct stimulation from TGF-β, 2) direct signaling from the ECM substratum and 3) additional stimulation elicited by alterations of integrin subunit expression induced by the presence of TGF-β and the ECM.

TGF-β is also intimately associated with the regulation of matrix degrading proteinases and their inhibitors (Sporn and Roberts, 1992; Attisano, et al., 1994). Differences observed between the various regions of the lens capsule may result from TGF-β induced production of MMPs. The degradation of the capsule by the MMPs could contribute to the heterogeneity of the lens capsule, aide in the migration of the lens cells or function to release matrix-bound growth factors which sustain terminal differentiation. To date, this function of TGF-β in the lens has not been examined.

In this report, we reveal that CLAP cells cultured in the presence of TGF-β secrete gelatinases with the molecular weights of 72, 77 and 92 kD. Based on the electrophoretic mobilities, the gelatinase activity detected at 72 and 92 kD suggests

that these enzymes may be MMP2 and MMP9, respectively. The identity of the 77 kD gelatinase is unknown. Gelatinase activity was inhibited by the inclusion of 10 mM EDTA in the incubation buffer. The lack of activity in the presence of a metal chelator confirms that the gelatinase bands are products of metalloproteinase activities. Identical results were obtained with TGF-β1 or TGF-β2 stimulation.

Gelatinase activity was only detected in media collected from cells cultured in the presence of TGF-β, suggesting that the production of gelatinases by CLAP cells is stimulated by TGF-β. Data presented here support the observation that gelatinase production by CLAP cells is specific to TGF-β stimulation and not the result of enhanced cellular attachment and spreading. Gelatinase activity was not induced by the presence of an ECM substratum which elicits similar degrees of cellular spreading as TGF-β stimulation. Our results are in agreement with the data reported by Salo, et al. (1991). Human keratinocytes were cultured on substrates of fibronectin, collagen type I or laminin, in the absence or presence of TGF-β. Expression of MMP2 and MMP9 was induced by TGF-β stimulation, independent of the type of ECM the cells were grown on. Gelatinase activity that could be induced by IGF-1, EGF or PDGF, never displayed the same pattern or intensity as the activity elicited by TGF-β. Therefore, gelatinase production is not a general response of cultured CLAP cells to growth factor stimulation and the secretion of all three gelatinases is specific to TGF-β stimulation.

Data presented here also demonstrates that TGF-β can stimulate CLAP cells to secrete proteins resembling uPA. The presence of uPA within the lenticular environment provides a key element in the integrated pathways possibly involved with

the modification of the lens capsule. uPA cleaves plasminogen to form plasmin, which then activates proMMPs and latent TGF-β (Vassalli, Sappino and Belin, 1991). Active TGF-β, in turn, stimulates the production of additional uPAs and induces the secretion of proMMPs. Active MMPs then function to modify the lens capsule which may assist lens cells migration and affect fiber cell differentiation. Therefore, uPA is an important component of TGF-β's matrix modifying activity.

Immunocytochemical localization of uPA within specific regions of the lens supports the idea that TGF-β may affect lens cell migration and differentiation. uPA was identified in the posterior lens capsule and in the equatorial epithelial cells, but could not be detected in the anterior capsule nor the central epithelium (Tripathi, Tripathi and Park, 1990). This distribution of uPA reflects functional differences in cellular motility between the different regions of the lens. Central epithelial cells are stationary and remain attached to the anterior lens capsule, therefore, uPA would not be expected to be expressed. Localization of uPA to the posterior capsule and equatorial epithelial cells suggests that these cells may be using the plasmin and MMP systems to produce a matrix capable of directing their migration. Since uPA synthesis is often controlled by TGF-β stimulation, this suggests that one possible function of TGF-β in the lens is establishing the highly localized expression of uPA.

In conclusion, we have shown that cultured and freshly isolated CLAP cells express TGF-β receptors type I and type II. Data presented here demonstrate that *in vitro* stimulation by TGF-β does not function to keep CLAP cells withdrawn from the cell cycle nor to promote the accumulation of differentiated characteristics. On the contrary, TGF-β functioned to enhance cellular proliferation. Proliferation seems to be

an inappropriate response to TGF-β, since CLAP cells *in vivo* remain withdrawn from the cell cycle. TGF-β stimulation induced the production of ECM components and MMPs. Therefore, TGF-β may play a role in the production of a heterogeneous lens capsule. TGF-β induced modification of the lens capsule may serve to direct cellular migration, to provide morphogenetic cues which participate in fiber cell differentiation or to modulate the activities of other growth factors. Future studies are required to define the interactions between growth factors, the production/degradation of the lens capule and the cellular responses elicited by both stimuli.

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ABSTRACT

ABSTRACT

POTENTIAL ROLES FOR TRANSFORMING GROWTH FACTOR-β DURING TERMINAL DIFFERENTIATION OF OCULAR LENS CELLS

by

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During terminal differentiation, lens fiber cells permanently withdraw from the cell cycle, migrate posteriorly along the lens capsule, become greatly elongated and eliminate all cellular organelles. Lens fiber differentiation may be controlled by various factors within the ocular environment. The lens capsule may influence lens fibers, since regions of the capsule differ in components and architecture. TGF-β is known as a regulator of both cell division and differentiation as well as a modulator of ECM production. Since TGF-β is found in the ocular environment and within lens cells, these functions of TGF-β in the lens were examined.

In the avian lens, post-mitotic cells destined to become lens fibers are located in a structure called the annular pad. TGF-β type I and type II receptors were identified by enhanced chemiluminescence in freshly isolated and cultured chicken lens annular pad (CLAP) cells. Immunoblot and metabolic labelling showed that TGF-β stimulation resulted in decreased synthesis of lens crystallins and no accumulation of a differentiation marker protein, phakinin. Measures of tritiated thymidine incorporation demonstrated that TGF-β stimulated, in a dose-dependent manner, cellular growth and

spreading.

The production of collagen type IV and fibronectin were detected in TGF-β stimulated CLAP cells using immunoblotting techniques and polyacrylamide gel electrophoresis. Substrates of collagen type IV, laminin or fibronectin stimulated increased thymidine incorporation and cellular spreading. TGF-β stimulation of cells cultured on an ECM substratum resulted in monolayer growth and a synergistic increase in thymidine incorporation.

Whether TGF-β modifies the lens capsule by stimulating CLAP cells to produce matrix degrading enzymes (MMPs) was examined by zymography. TGF-β induced MMP activity corresponding to MMP2, MMP9 and an unknown MMP at approximate 77 kD molecular weight.

Thus, TGF-β does not function to keep CLAP cells withdrawn from the cell cycle or to promote fiber cell differentiation. TGF-β does induce CLAP cells to produce ECM proteins and matrix degrading enzymes. Thus, TGF-β may inhibit mitosis *in vivo*, but more importantly, it may influence the composition of the lens capsule.

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1991	Fight for Sight - Independent Order of Odd Fellows Student Fellowship
1993-1996	Graduate Student Council - Student Representative
1994-1995	Midwest Eye-Banks and Transplantation Center Award

Publications:

A. Banerjee, **D.M. Richiert**, K. Emanuel, A.K. Singh and M. Bagchi. Studies on the possible role of vitreous humor on the protein synthesis and morphology of the organ cultured adult lens. II. Epithelial cells. Biochem. Biophys. Acta 1076:330-336, 1991.

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